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(54) VARIANT PHOSPHOENOLPYRUVATE CARBOXYLASE, GENE THEREOF, AND PROCESS FOR PRODUCING AMINO ACID

(57) A variant phosphoenolpyruvate carboxylase that is not substantially inhibited by aspartic acid is produced by introducing a variant phosphoenolpyruvate carboxylase gene, such as one wherein the 625th glutamic acid residue from the N-terminus of the carboxylase has been replaced by a lysine residue or one wherein the 438th arginine residue has been replaced by a cysteine residue, into *Escherichia coli* or a coryneform bacterium. An amino acid can efficiently be produced by using this carboxylase.

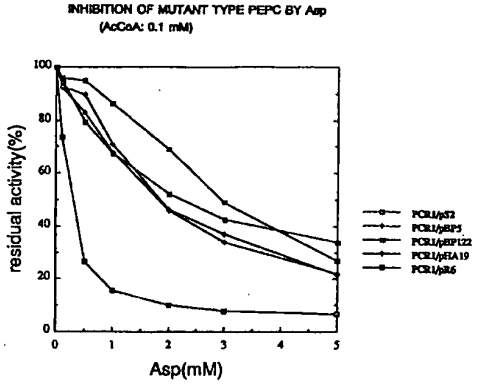


Fig. 9

EP 0 723 011 A1

Description

TECHNICAL FIELD

The present invention relates to a mutant phosphoenolpyruvate carboxylase, a gene coding for it, and a production method of an amino acid, and in particular relates to a gene having mutation to desensitize feedback inhibition by aspartic acid, and utilization thereof.

BACKGROUND ART

Phosphoenolpyruvate carboxylase is an enzyme which is found in almost all bacteria and all plants. The role of this enzyme resides in biosynthesis of aspartic acid and glutamic acid, and supply of C4 dicarboxylic acid to the citric acid cycle for maintaining its turnover. However, in the fermentative production of an amino acid using a microorganisms, there have been few reports on effects of this enzyme has not been made clear (Atsushi Yokota and Isamu Shiio, Agric. Biol. Chem., 52, 455-463 (1988), Josef Cremer et al., Appl. Environ. Microbiol.,57, 1746-1752 (1991), Petra, G. Peters-Weintisch, FEMS Microbiol. Letters, 112, 269-274 (1993)).

By the way, the amino acid is a compound which universally exists in cells as components of proteins, however, for the sake of economic energy metabolism and substance metabolism, its production is strictly controlled. This control is principally feedback control, in which the final product of a metabolic pathway inhibits the activity of an enzyme which catalyzes the earlier step of the pathway. Phosphoenolpyruvate carboxylase also undergoes various regulations in expression of its activity.

For example, in the case of phosphoenolpyruvate carboxylase of microorganisms belonging to the genus Corynebacterium or the genus Escherichia, the activity is inhibited by aspartic acid. Therefore, the aforementioned amino acid biosynthesis, in which phosphoenolpyruvate carboxylase participates, is also inhibited by aspartic acid.

In the prior art, various techniques have been developed for efficient production in amino acid fermentation, and fermentative production has been carried out for leucine, isoleucine, tryptophan, phenylalanine and the like by using mutant strains converted to be insensitive to feedback control. However, there has been known neither mutant phosphoenolpyruvate carboxylase converted to be insensitive to inhibition by aspartic acid, nor attempt to utilize it for fermentative production of amino acids of the aspartic acid family and the glutamic acid family.

On the other hand, ppc gene, which is a gene coding for phosphoenolpyruvate carboxylase of Escherichia coli, has been already cloned, and also determined for its nucleotide sequence (Fujita, N., Miwa, T., Ishijima, S., Izui, K. and Katsuki, H., J. Biochem., 95, 909-916 (1984)). However, there is no report of a mutant in which inhibition by aspartic acid is desensitized.

The present invention has been made from the aforementioned viewpoint, an object of which is to provide a mutant phosphoenolpyruvate carboxylase with substantially desensitized feedback inhibition by aspartic acid, a gene conding for it, and a utilization method thereof.

DISCLOSURE OF THE INVENTION

As a result of diligent investigation in order to achieve the aforementioned object, the present inventors have found that the inhibition by aspartic acid is substantially desensitized by replacing an amino acid at a specified site of phosphoenolpyruvate carboxylase of Escherichia coli with another amino acid, succeeded in obtaining a gene coding for such a mutant enzyme, and arrived at completion of the present invention.

Namely, the present invention lies in a mutant phosphoenolpyruvate carboxylase, which originates from a microorganism belonging to the genus Escherichia, and has mutation to desensitize feedback inhibition by aspartic acid, and a DNA sequence coding for the mutant phosphoenolpyruvate carboxylase.

The present invention further provides microorganisms belonging to the genus Escherichia or coryneform bacteria harboring the DNA fragment, and a method of producing an amino acid wherein any of these microorganisms is cultivated in a preferable medium, and the amino acid selected from L-lysine, L-threonine, L-methionine, L-isoleucine, L-glutamic acid, L-arginine and L-proline is separated from the medium.

Incidentally, in this specification, the DNA sequence coding for the mutant phosphoenolpyruvate carboxylase, or a DNA sequence containing a promoter in addition thereto is occasionally merely referred to as "DNA sequence of the present invention", "mutant gene" or "phosphoenolpyruvate carboxylase gene."

The present invention will be explained in detail hereinafter.

(1) Mutant phosphoenolpyruvate carboxylase

The mutant phosphoenolpyruvate carboxylase of the present invention (hereinafter simply referred to as "mutant enzyme") lies in the phosphoenolpyruvate carboxylase of the microorganism belonging to the genus Escherichia, which has mutation to desensitize the feedback inhibition by aspartic acid.

Such mutation may be any one provided that the aforementioned feedback inhibition is substantially desensitized without losing the enzyme activity of the phosphoenolpyruvate carboxylase, for which there may be exemplified mutation which, when a mutant phosphoenolpyruvate carboxylase having the mutation is allowed to exist in cells of a microorganism belonging to the genus Escherichia, gives the cells resistance to a compound having the following properties:

it exhibits a growth inhibitory action against a microorganism belonging to the genus Escherichia which produces a wild type phosphoenolpyruvate carboxylase;

the aforementioned growth inhibitory action is recovered by existence of L-glutamic acid or L-aspartic acid; and

it inhibits wild type phosphoenolpyruvic carboxylase activity.

More concretely, there may be exemplified, as counted from the N-terminus of the phosphoenolpyruvate carboxylase:

(1) mutation to replace 625th glutamic acid with lysine;
(2) mutation to replace 222th arginine with histidine and 223th glutamic acid with lysine, respectively;
(3) mutation to replace 288th serine with phenylalanine, 289th glutamic acid with lysine, 551th methionine with isoleucine and 804th glutamic acid with lysine, respectively;
(4) mutation to replace 867th alanine with threonine;
(5) mutation to replace 438th arginine with cysteine; and
(6) mutation to replace 620th lysine with serine.

Incidentally, as the phosphoenolpyruvate carboxylase of the microorganism belonging to the genus Escherichia, an amino acid sequence, which is deduced from a phosphoenolpyruvate carboxylase gene of Escherichia coli (Fujita, N., Miwa, T., Ishijima, S., Izui, K. and Katsuki, H., J. Biochem., 95, 909-916 (1984)), is shown in SEQ ID NO:2 in the Sequence listing. In addition, an entire nucleotide sequence of a plasmid pT2, which contains the phosphoenolpyruvate carboxylase gene of Escherichia coli, is shown in SEQ ID NO:1 together with the amino acid sequence.

The aforementioned mutant enzymes are encoded by DNA sequences of the present invention described below, which are produced by expressing the DNA sequences in Escherichia coli and the like.

(2) DNA sequence of the present invention and microorganisms harboring the same

The DNA sequence of the present invention is DNA sequences coding for the aforementioned mutant enzymes, and has mutation to desensitize feedback inhibition of phosphoenolpyruvate carboxylase by aspartic acid in coding regions in DNA fragments coding for phosphoenolpyruvate carboxylase of the microorganism belonging to the genus Escherichia.

Concretely, there may be exemplified a DNA Sequence coding for the phosphoenolpyruvate carboxylase having the mutation of any one of the aforementioned (1) to (6), for example, with respect to the nucleotide sequence of SEQ ID NO:1, there may be exemplified a DNA sequence having any one of:

i) mutation to convert GAA of base Nos. 2109-2111 into AAA or AAG;
ii) mutation to convert CGC of base Nos. 900-902 into CAT or CAC, and GAA of 903-905 into AAA or AAG, respectively;
iii) mutation to convert TCT of base Nos. 1098-1100 into TTT or TTC, GAA of 1101-1103 into AAA or AAG, ATG of 1887-1889 into ATT, ATC or ATA, and GAA of 2646-2648 into AAA or AAG, respectively;
iv) mutation to convert GCG of 2835-2837 into any one of ACT, ACC, ACA and ACG; and
v) mutation to convert CGT of 1548-1550 into TGT or TGC; and
vi) mutation to convert AAA of 2094-2096 into TCT, TCC, TCA or TCG.

Such a mutant gene is obtained such that a recombinant DNA, which is obtained by ligating a phosphoenolpyruvate carboxylase gene as a wild type enzyme gene or having another mutation with a vector DNA adaptable to a host, is subjected to a mutation treatment, to perform screening from transformants by the recombinant DNA. Alternatively, it is also acceptable that a microorganism which produces a wild type enzyme is subjected to a mutation treatment, a mutant strain which produces a mutant enzyme is created, and then a mutant gene is screened from the mutant strain. For the mutation treatment of the recombinant DNA, hydroxylamine and the like may be used. Further, when an microorganism itself is subjected to a mutation treatment, a drug or a method usually used for artificial mutation may be used.

Further, in accordance with methods such as the Overlapping Extension method (Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R., Gene, 77, 51-59 (1989)), the site specific mutation method (Kramer, W. and Frits, H. J., Meth. in Enzymol., 154, 350 (1987); Kunkel, T. A. et al., Meth. in Enzymol., 154, 367 (1987)) and the like, the aforementioned mutant gene can be also obtained by introducing mutation such as amino acid replacement, insertion, deletion and the like into a phosphoenolpyruvate carboxylase gene as a wild type enzyme gene or having another mutation. These methods are based on a principle that a non-mutated gene DNA is used as a template, and a synthetic DNA containing a mismatch at a mutation point is used as one of primers so as to synthesize complemental strands of the aforementioned gene DNA, thereby mutation is introduced. By using these methods, it is possible to cause intended mutation at an aimed site.

Alternatively, a sequence, which has restriction enzyme cleavage ends at both termini and includes both sides of a mutation point, is synthesized, and exchanged for a corresponding portion of a non-mutated gene, thereby mutation can be introduced (cassette mutation method).

The phosphoenolpyruvate carboxylase gene, which is the wild type enzyme gene or has another mutation to be used for introduction of mutation, may be any one provided that it is a gene coding for the phosphoenolpyruvate carboxylase of the microorganism belonging to the genus Escherichia, which is preferably determined for its base sequence and cloned. When it has not been cloned, a DNA fragment containing the gene can be amplified and isolated by using the PCR method and the like, followed by using a suitable vector to achieve cloning.

As the gene as described above, for example, there may be exemplified a gene of Escherichia coli having been cloned and determined for its base sequence (Fujita, N., Miwa, T., Ishijima, S., Izui, K. and Katsuki, H., J. Biochem., 95, 909-916 (1984)). The sequence in the coding region of this gene is as shown in SEQ ID NO: 1 (base Nos. 237-2888).

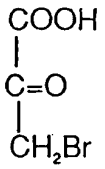
Screening of a host harboring the mutant gene can be performed by using an analog compound of aspartic acid. The analog compound preferably has the following properties. Namely, it exhibits a growth inhibitory action against a microorganism belonging to the genus Escherichia which produces a wild type phosphoenolpyruvate carboxylase, the aforementioned growth inhibitory action is recovered by existence of L-glutamic acid or L-aspartic acid, and it inhibits wild type phosphoenolpyruvate carboxylase activity.

If a mutant strain beeing resistant to the analog compound mentioned above is selected from microorganism belonging to the genus Escherichia, for example, Escherichia coli HB101 producing wild type phosphoenolpyruvate carboxylase using inhibition of growth of the microorganism as an index, it is much likely to obtain a host microorganism which produces phosphoenolpyruvate carboxylase with desensitized feedback inhibition by aspartic acid.

It is proposed, as a general structure of an inhibitor of phosphoenolpyruvate carboxylase, that a C4 dicarboxylic acid structure is essentially provided. From such a viewpoint, various compounds were subjected to screening by the present inventors. Escherichia coli HB101 was cultivated in an LB medium, and transferred to M9 media (containing 20 μg/ml of thiamine and 3 μg/ml of each of Leu and Pro) containing any one of DL-2-amino-4-phosphonobutyric acid, bromosuccinic acid, meso-2,3-dibromosuccinic acid, 2,2-difluorosuccinic acid, 3-bromopyruvic acid, α-ketobutyric acid, α-ketoadipinic acid、DL-threo-β-hydroxyaspartic acid、L-aspartic acid β-metyl ester、α-metyl-DL-aspartic acid, 2,3-diaminosuccinic acid or aspartic acid-β-hydrazide, and absorbance of the medium was measured at 660 nm with the passage of time, thereby growth was monitored.

Further, when these compounds were present at their growth inhibitory concentrations, it was investigated whether or not the inhibition was recovered by addition of nucleic acids (each of uridine, adenosine: 10 mg/dl), glutamic acid or amino acids of the aspartic acid family (Asp: 0.025 %, each of Met, Thr, Lys: 0.1 %).

As a result, three compounds: 3-bromopyruvate (3BP) (1), aspartate-β-hydrazide (AHY) (2), and DL-threo-β-hydroxyaspartate (βHA) (3) were selected.



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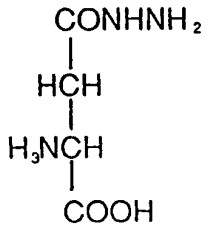

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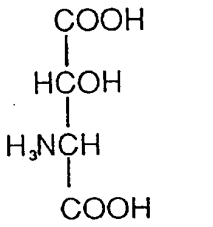

...(3)

Growth inhibition of Escherichia coli by these analog compounds is shown in Figs. 1-3. Further, growth recovery of Escherichia coli, in the case of addition of the aforementioned inhibition recovering substances alone or as a mixture of 2 species or 3 species, is shown in Figs. 4-6. In addition, as a control, growth in the case of addition of the inhibition recovering substance in the absence of the inhibitory substance is shown in Fig. 7. Incidentally, in Figs. 4-7, additives 1, 2 and 3 indicate nucleic acids, glutamic acid or amino acids of the aspartic acid family, respectively.

Further, inhibition of activity by the analog compound on phosphoenolpyruvate carboxylase was investigated. Crude enzyme was prepared from an Escherichia coli HB101 strain in accordance with a method described in The Journal of Biochemistry, Vol. 67, No. 4 (1970), and enzyme activity was measured in accordance with a method described in Eur. J. Biochem., 202, 797-803 (1991).

Escherichia coli HB101 cultivated in an LB medium was disrupted, and a suspension was centrifuged to obtain a supernatant which was used as a crude enzyme solution. Measurement of enzyme activity was performed by measuring decrease in absorbance at 340 nm while allowing acetyl-coenzyme A known to affect the activity to exist at a concentration of 0.1 mM in a measurement system containing 2 mM potassium phosphoenolpyruvate, 0.1 mM NADH, 0.1 M Tris-acetate (pH 8.5), 1.5 U malate dehydrogenase and crude enzyme. Results are shown in Fig. 8.

According to the results as above, it is apparent that the aforementioned three compounds inhibit growth of Escherichia coli, this inhibition cannot be recovered by nucleic acids alone, but the inhibition can be recovered by addition of glutamic acid or amino acids of the aspartic acid family. Therefore, these analog compounds were postulated to be selective inhibitors of phosphoenolpyruvate carboxylase. As shown in Examples described below, by using these compounds, the present invention has succeeded in selection of an Escherichia coli which produces the mutant phosphoenolpyruvate carboxylase.

When a transformant having an aimed mutant enzyme gene is screened by using the aforementioned compounds, and a recombinant DNA is recovered, then the mutant enzyme gene is obtained. Alternatively, in the case of a mutation treatment of an microorganism itself, when a mutant strain having an aimed mutant enzyme gene is screened by using the aforementioned compounds, a DNA fragment containing the aimed mutant enzyme gene is isolated from the strain, and it is ligated with a suitable vector, then the mutant enzyme gene is obtained.

On the other hand, as a result of diligent investigation by the present inventors taking notice of importance of an arginine residue in an aspartate binding protein of Escherichia coli (Krikos, A., Mouth, N., Boyd, A. and Simon, M. I. Cell, 33, 615-622 (1983), Mowbray, S. L and Koshland, D. E. J. Biol. Chem., 264, 15638-15643 (1990), Milburn, M. V., Prive, G. G., Milligan, D. L., Scott, W. G., Yeh, J., Jancarik, J., Koshland, D. E. and Kim, S. H., Science, 254, 1342-1347 (1991)), it has been found that inhibition by aspartic acid is substantially desensitized by converting 438th arginine of phosphoenolpyruvate carboxylase into cysteine. In order to convert 438th arginine into cysteine, a codon of 438th

arginine of a gene coding for phosphoenolpyruvate carboxylase may be converted into a codon of cysteine. For example, in SEQ ID NO:1, CGT of nucleotide numbers of 1548-1550 may be converted into TGT or TGC.

Further, th present inventors performed chemical modification of lysine residues of phosphoenolpyruvate carboxylase by using 2,4,6-trinitrobenzenesulfonic acid (TNBS) which is a compound to chemically modify lysine residues of a protein. During modification reaction, malic acid capable of serving as an inhibitor of phosphoenolpyruvate carboxylase was allowed to exist together. Namely, it was assumed that a lysine residue in the vicinity of a binding position of phosphoenolpyruvate carboxylase would be protected by bound malic acid and not be subjected to chemical modification. As a result, it was suggested that a 620th lysine residue was important for malic acid to bind phosphoenolpyruvate carboxylase, and it was found that the feedback inhibition by aspartic acid was desensitized while maintaining the enzyme activity of phosphoenolpyruvate carboxylase by converting the 620th lysine residue into a serine residue. In order to convert the 620th lysine residue into the serine residue, a codon of 620th lysine of the gene coding for phosphoenolpyruvate carboxylase may be converted into a codon of serine. For example, in SEQ ID NO:1, AAA having nucleotide numbers of 2094-2096 may be replaced with TCT, TCC, TCA, TCG, AGT or AGC.

In accordance with methods such as the Overlapping Extension method (Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R., Gene, 77, 51-59 (1989)), the site specific mutation method (Kramer, W. and Frits, H. J., Meth. in Enzymol., 154, 350 (1987); Kunkel, T. A. et al., Meth. in Enzymol., 154, 367 (1987)) and the like, conversion of the codon can be also achieved by introducing mutation such as amino acid replacement, insertion, deletion and the like into a phosphoenolpyruvate carboxylase gene as a wild type enzyme gene or having another mutation. These methods are based on a principle that a non-mutated gene DNA is used as a template, and a synthetic DNA containing a mismatch at a mutation point is used as one of primers so as to synthesize complemental strands of the aforementioned gene DNA, thereby mutation is introduced. By using these methods, it is possible to cause intended mutation at an aimed site.

Alternatively, a sequence, which has restriction enzyme cleavage ends at both termini and contains both sides of a mutation point, is synthesized, and exchanged for a corresponding portion of a non-mutated gene, thereby mutation can be introduced (cassette mutation method).

The DNA fragment coding for the phosphoenolpyruvate carboxylase with mutation introduced as described above is expressed by using a suitable host-vector system, thereby it is possible to produce a mutant enzyme. Alternatively, even by performing transformation by integrating the DNA fragment of the present invention into a host chromosomal DNA, an aimed mutant enzyme can be produced.

As the host, there may be exemplified microorganisms belonging to the genus Escherichia, for example, Escherichia coli, coryneform bacteria and the like. The coryneform bacteria include bacteria belonging to the genus Corynebacterium, bacteria belonging to the genus Brevibacterium having been hitherto classified into the genus Brevibacterium but being united as bacteria belonging to the genus Corynebacterium at present, and bacteria belonging to the genus Brevibacterium closely related to bacteria belonging to the genus Corynebacterium. Incidentally, hosts which are preferable for amino acid production will be described below.

On the other hand, as the vector DNA, a plasmid vector is preferable, and those capable of self-replication in a host cell are preferable. When the host is Escherichia coli, for example, pUC19, pUC18, pBR322, pHSG299, pHSG399, RSF1010 and the like are exemplified. Alternatively, a vector of phage DNA can be also utilized.

Further, when the host is the coryneform bacteria, vectors which can be used and hosts which harbor them are exemplified below. Incidentally, deposition numbers of international depositories are shown in parentheses.

pAJ655	Escherichia coli AJ11882 (FERM BP-136)
	Corynebacterium glutamicum SR8201 (ATCC 39135)
pAJ1844	Escherichia coli AJ11883 (FERM BP-137)
	Corynebacterium glutamicum SR8202 (ATCC 39136)
pAJ611	Escherichia coli AJ11884 (FERM BP-138)
pAJ3148	Corynebacterium glutamicum SR8203 (ATCC 39137)
pAJ440	Bacillus subtilis AJ11901 (FERM BP-140)

These vectors may be obtained from the deposited microorganisms as follows. Cells collected at the logarithmic growth phase are subjected to bacteriolysis by using lysozyme and SDS, and centrifuged at 30000 x g to obtain a supernatant solution from a lysate, to which polyethylene glycol is added to perform separation and purification of the vectors by means of cesium chloride-ethidium bromide equilibrium density gradient centrifugation.

In order to transform Escherichia coli with a recombinant vector obtained by inserting the DNA sequence of the present invention into the aforementioned vector, it is possible to use a method usually used for transformation of Escherichia coli, such as a method in which cells are treated with calcium chloride to enhance permeability of DNA (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1977)) and the like.

Further, as a method for transforming the coryneform bacteria, there is the aforementioned method in which cells are treated with calcium chloride, or a method in which incorporation is performed at a specified growth period in which

cells can incorporate DNA (report in relation to Bacillus subtilis by Duncan, C. H. at al.). Further, incorporation into bacterial cells can be achieved by forming protoplasts or spheroplasts of DNA recipients which easily incorporate plasmid DNA. These are known for Bacillus subtilis, Actinomyces and yeast (Chang, S. et al., Molec. Gen. Genet., 168, 111 (1979), Bibb et al., Nature, 274, 398 (1978), Hinnen, A. et al., Proc. Natl. Acad. Sci. USA, 75 1929 (1978)). Additionally, a method for transforming coryneform bacteria is disclosed in Japanese Patent Laid-open No. 2-207791.

In order to express the DNA sequence of the present invention in the aforementioned hosts, a promoter such as lac, trp, PL and the like which efficiently works in microorganisms may be used, or when the DNA sequence of the present invention contains a promoter of the phosphoenolpyruvate carboxylase gene, it may be used as it is. Alternatively, when the coryneform bacterium is used as the host, it is also possible to use a known trp promoter originating from a bacterium belonging to the genus Brevibacterium (Japanese Patent Laid-open No. 62-244382) and the like.

Further, as described above, it is acceptable that the DNA sequence of the present invention is inserted into the vector DNA capable of self-replication and introduced into the host to allow the host to harbor it as a plasmid, and it is also acceptable that the DNA sequence of the present invention is integrated into a chromosome of an microorganism by means of a method using transposon (Berg, D. E. and Berg, C. M., Bio/Technol., 1, 417 (1983)), Mu phage (Japanese Patent Laid-open No. 2-109985) or homologous recombination (Experiments in Molecular Genetics, Cold Spring Harbor Lab. (1972)). In addition, in order to integrate the DNA of the present invention into the coryneform bacteria, it is possible to utilize a temperature-sensitive plasmid disclosed in Japanese Patent Laid-open No. 5-7491.

When the microorganism transformed with the DNA sequence of the present invention as described above is cultivated, and this DNA sequence is expressed, then a mutant enzyme is obtained. It becomes apparent, by measuring the activity by adding aspartic acid to an enzyme reaction system, whether or not the mutant enzyme thus obtained has desensitized feedback inhibition by aspartic acid. It is possible for the measurement of the enzyme activity to use a spectrometric method (Yoshinage, T., Izui, K. and Katsuki, H., J. Biochem., 68, 747-750 (1970)) and the like.

Further, the DNA sequence of the present invention codes for the mutant enzyme in which feedback inhibition by aspartic acid is desensitized, so that the microorganism harboring this DNA sequence can be utilized for efficient fermentative production of amino acids of the aspartic acid family and the glutamic acid family as described below.

Escherichia coli AJ12907, AJ12908, AJ12909 and AJ12910 harboring the mutant enzyme genes obtained in Examples described below are deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan; zip code 305) on August 3, 1993 under the deposition numbers of FERM P-13774, FERM P-13775, FERM P-13776 and FERM P-13777, transferred from the original deposition to international deposition based on Budapest Treaty on July 11, 1994 and has been deposited as deposition numbers of FERM BP-4734、FERM BP-4735、FERM BP-4736、FERM BP-4737, respectively in this order.

(3) Production method of amino acids

Amino acids can be produced by cultivating the microorganism harboring the DNA sequence of the present invention in a preferable medium, and separating generated amino acids. As such amino acids, there may be exemplified L-lysine, L-threonine, L-methionine, L-isoleucine, L-glutamic acid, L-arginine and L-proline.

Preferable hosts into which the DNA sequence of the present invention is introduced to be used for production of each of the amino acids, and a cultivation method will be exemplified below.

(1) Hosts preferable for the amino acid production method of the present invention

(i) Hosts preferable for L-lysine production

As the host to be used for L-lysine production according to the present invention, there may be exemplified bacteria belonging to the genus Escherichia, preferably L-lysine-producing Escherichia coli. Concretely, a mutant strain having resistance to a lysine analog can be exemplified. Such a lysine analog is those which inhibit growth of microorganisms belonging to the genus Escherichia, however, the suppression is totally or partially desensitized provided that L-lysine co-exits in the medium. For example, there are oxalysine, lysine hydroxamate, S-(2-aminoethyl)-cysteine (hereinafter abbreviated as "AEC"), γ-methyllysine, α-chlorocaprolactam and the like. Mutant strains having resistance to these lysine analogs can be obtained by applying an ordinary artificial mutation treatment to microorganisms belonging to the genus Escherichia. Concretely, as a bacterial strain to be used for L-lysine production, there may be exemplified Escherichia coli AJ11442 (deposited as FERM P-5084, see lower-left column on page 471 in Japanese Patent Laid-open No. 56-18596).

On the other hand, various artificial mutant strains of coryneform bacteria which have been used as L-lysine-producing bacteria can be used for the present invention. Such artificial mutant strains are as follows: AEC resistant mutant strain; mutant strain which requires amino acid such as L-homoserine for its growth (Japanese Patent Publication Nos. 48-28078 and 56-6499); mutant strain which exhibits resistance to AEC and requires amino acid such as L-leucine, L-

homoserine, L-proline, L-serine, L-arginine, L-alanine, L-valine and the like (United States Patent Nos. 3708395 and 3825472); L-lysine-producing mutant strain which exhibits resistance to DL-α-amino-ε-caprolactam, α-amino-lauryl-lactam, quinoid and N-lauroylleucine; L-lysine-producing mutant strain which exhibits resistance to an inhibitor of oxaloacetate decarboxylase or respiratory system enzyme (Japanese Patent Laid-open Nos. 50-53588, 50-31093, 52-102498, 53-86089, 55-9783, 55-9759, 56-32995 and 56-39778, and Japanese Patent Publication Nos. 53-43591 and 53-1833); L-lysine-producing mutant strain which requires inositol or acetic acid (Japanese Patent Laid-open Nos. 55-9784 and 56-8692); L-lysine-producing mutant strain which exhibits sensitivity to fluoropyruvate or temperature not less than 34 °C (Japanese Patent Laid-open Nos. 55-9783 and 53-86090); and mutant strain of Brevibacterium or Corynebacterium which exhibits resistance to ethylene glycol and produces L-lysine (see United States Patent Application Serial No. 333455).

Followings are exemplified as concrete coryneform bacteria to be used for lysine production:

Brevibacterium lactofermentum AJ12031 (FERM-BP277), see page 525 in Japanese Patent Laid-open No. 60-62994;

Brevibacterium lactofermentum ATCC 39134, see lower-right column on page 473 in Japanese Patent Laid-open No. 60-62994;

Brevibacterium lactofermentum AJ3463 (FERM-P1987), see Japanese Patent Publication No. 51-34477.

In addition, wild strains of coryneform bacteria described below can be also used for the present invention in the same manner.

Corynebacterium acetoacidophilum
ATCC 13870
Corynebacterium acetoglutamicum
ATCC 15806
Corynebacterium callunae
ATCC 15991
Corynebacterium glutamicum
ATCC 13032
ATCC 13060
(Brevibacterium divaricatum)
ATCC 14020
(Brevibacterium lactofermentum)
ATCC 13869
(Corynebacterium lilium)
ATCC 15990
Corynebacterium melassecola
ATCC 17965
Brevibacterium saccharolyticum
ATCC 14066
Brevibacterium immariophilum
ATCC 14068
Brevibacterium roseum
ATCC 13825
Brevibacterium flavum
ATCC 13826
Brevibacterium thiogenitalis
ATCC 19240
Microbacterium ammoniaphilum
ATCC 15354

(ii) Hosts preferable for L-threonine production

Escherichia coli B-3996 (RIA 1867), see Japanese Patent Laid-open No. 3-501682 (PCT);

Escherichia coli AJ12349 (FERM-P9574), see upper-left column on page 887 in Japanese Patent Laid-open No. 2-458;

Escherichia coli AJ12351 (FERM-P9576), see lower-right column on page 887 in Japanese Patent Laid-open No. 2-458;

Escherichia coli AJ12352 (FERM P-9577), see upper-left column on page 888 in Japanese Patent Laid-open No. 2-458;

Escherichia coli AJ11332 (FERM P-4898), see upper-left column on page 889 in Japanese Patent Laid-open No.

2-458;

Escherichia coli AJ12350 (FERM P-9575), see upper-left column on page 889 in Japanese Patent Laid-open No. 2-458;

Escherichia coli AJ12353 (FERM P-9578), see upper-right column on page 889 in Japanese Patent Laid-open No. 2-458;

Escherichia coli AJ12358 (FERM P-9764), see upper-left column on page 890 in Japanese Patent Laid-open No. 2-458;

Escherichia coli AJ12359 (FERM P-9765), see upper-left column on page 890 in Japanese Patent Laid-open No. 2-458;

Escherichia coli AJ11334 (FERM P-4900), see column 6 on page 201 in Japanese Patent Publication No. 1-29559;

Escherichia coli AJ11333 (FERM P-4899), see column 6 on page 201 in Japanese Patent Publication No. 1-29559;

Escherichia coli AJ11335 (FERM P-4901), see column 7 on page 202 in Japanese Patent Publication No. 1-29559.

Following bacterial strains are exemplified as the coryneform bacteria:

Brevibacterium lactofermentum AJ11188 (FERM P-4190), see upper-right column on page 473 in Japanese Patent Laid-open No. 60-87788;

Corynebacterium glutamicum AJ11682 (FERM BP-118), see column 8 on page 230 in Japanese Patent Publication No. 2-31956;

Brevibacterium flavum AJ11683 (FERM BP-119), see column 10 on page 231 in Japanese Patent Publication No. 2-31956.

(iii) Hosts preferable for L-methionine production

Following bacterial strains are exemplified for L-methionine production:

Escherichia coli AJ11457 (FERM P-5175), see upper-right column on page 552 in Japanese Patent Laid-open No. 56-35992;

Escherichia coli AJ11458 (FERM P-5176), see upper-right column on page 552 in Japanese Patent Laid-open No. 56-35992;

Escherichia coli AJ11459 (FERM P-5177), see upper-right column on page 552 in Japanese Patent Laid-open No. 56-35992;

Escherichia coli AJ11539 (FERM P-5479), see lower-left column on page 435 in Japanese Patent Laid-open No. 56-144092;

Escherichia coli AJ11540 (FERM P-5480), see lower-left column on page 435 in Japanese Patent Laid-open No. 56-144092;

Escherichia coli AJ11541 (FERM P-5481), see lower-left column on page 435 in Japanese Patent Laid-open No. 56-144092;

Escherichia coli AJ11542 (FERM P-5482), see lower-left column on page 435 in Japanese Patent Laid-open No. 56-144092.

(iv) Hosts preferable for L-aspartic acid production

Following bacterial strains are exemplified for L-aspartic acid production:

Brevibacterium flavum AJ3859 (FERM P-2799), see upper-left column on page 524 in Japanese Patent Laid-open No. 51-61689;

Brevibacterium lactofermentum AJ3860 (FERM P-2800), see upper-left column on page 524 in Japanese Patent Laid-open No. 51-61689;

Corynebacterium acetoacidophilum AJ3877 (FERM-P2803), see upper-left column on page 524 in Japanese Patent Laid-open No. 51-61689;

Corynebacterium glutamicum AJ3876 (FERM P-2802), see upper-left column on page 524 in Japanese Patent Laid-open No. 51-61689.

(v) Hosts preferable for L-isoleucine production

Escherichia coli KX141 (VKPM-B4781) (see 45th paragraph in Japanese Patent Laid-open No. 4-33027) is exemplified as the bacteria belonging to the genus Escherichia, and Brevibacterium lactofermentum AJ12404 (FERM P-10141) (see lower-left column on page 603 in Japanese Patent Laid-open No. 2-42988) and Brevibacterium flavum

AJ12405 (FERM P-10142) (see lower-left column on page 524 in Japanese Patent Laid-open No. 2-42988) are exemplified as the coryneform bacteria.

(vi) Hosts preferable for L-glutamic acid production

Following bacterial strains are exemplified as the bacteria belonging to the genus Escherichia:
Escherichia coli AJ12628 (FERM P-12380), see French Patent Publication No. 2 680 178 (1993);
Escherichia coli AJ12624 (FERM P-12379), see French Patent Publication No. 2 680 178 (1993).
Following bacterial strains are exemplified as the coryneform bacteria:
Brevibacterium lactofermentum AJ12745 (FERM BP-2922), see lower-right column on page 561 in Japanese Patent Laid-open No. 3-49690;
Brevibacterium lactofermentum AJ12746 (FERM BP-2923), see upper-left column on page 562 in Japanese Patent Laid-open No. 3-49690;
Brevibacterium lactofermentum AJ12747 (FERM BP-2924), see upper-left column on page 562 in Japanese Patent Laid-open No. 3-49690;
Brevibacterium lactofermentum AJ12748 (FERM BP-2925), see upper-left column on page 562 in Japanese Patent Laid-open No. 3-49690;
Brevibacterium flavum ATCC 14067, see Table 1 on page 3 in Japanese Patent Laid-open No. 5-3793;
Corynebacterium glutamicum ATCC 21492, see Table 1 on page 3 in Japanese Patent Laid-open No. 5-3793.

(vii) Hosts preferable for L-arginine production

Following bacterial strains are exemplified as the bacteria belonging to the genus Escherichia:
Escherichia coli AJ11593 (FERM P-5616), see upper-left column on page 468 in Japanese Patent Laid-open No. 57-5693;
Escherichia coli AJ11594 (FERM P-5617), see upper-right column on page 468 in Japanese Patent Laid-open No. 57-5693.
Following bacterial strains are exemplified as the coryneform bacteria:
Brevibacterium flavum AJ12144 (FERM P-7642), see column 4 on page 174 in Japanese Patent Publication No. 5-27388;
Corynebacterium glutamicum AJ12145 (FERM P-7643), see column 4 on page 174 in Japanese Patent Publication No. 5-27388;
Brevibacterium flavum ATCC 21493, see Table 1 on page 3 in Japanese Patent Laid-open No. 5-3793;
Corynebacterium glutamicum ATCC 21659, see Table 1 on page 3 in Japanese Patent Laid-open No. 5-3793.

(viii) Hosts preferable for L-proline production

Following bacterial strains are exemplified as the bacteria belonging to the genus Escherichia:
Escherichia coli AJ11543 (FERM P-5483), see lower-left column on page 435 in Japanese Patent Laid-open No. 56-144093;
Escherichia coli AJ11544 (FERM P-5484), see lower-left column on page 435 in Japanese Patent Laid-open No. 56-144093.
Following bacterial strains are exemplified as the coryneform bacteria:
Brevibacterium lactofermentum AJ11225 (FERM P-4370), see upper-left column on page 473 in Japanese Patent Laid-open No. 60-87788;
Brevibacterium flavum AJ11512 (FERM P-5332), see column 2 on page 185 in Japanese Patent Publication No. 62-36679;
Brevibacterium flavum AJ11513 (FERM P-5333), see column 2 on page 185 in Japanese Patent Publication No. 62-36679;
Brevibacterium flavum AJ11514 (FERM P-5334), see column 2 on page 185 in Japanese Patent Publication No. 62-36679;
Corynebacterium glutamicum AJ11522 (FERM P-5342), see column 2 on page 185 in Japanese Patent Publication No. 62-36679;
Corynebacterium glutamicum AJ11523 (FERM P-5343), see column 2 on page 185 in Japanese Patent Publication No. 62-36679.

(2) Cultivation method

The method for cultivating the aforementioned hosts is not especially different from a cultivation method for amino acid-producing microorganisms in the prior art. Namely, an ordinary medium is used containing a carbon source, a nitrogen source and inorganic ions, and optionally organic trace nutrients such as amino acids, vitamins and the like.

As the carbon source, glucose, sucrose, lactose and the like, as well as starch hydrolysate, whey, molasses and the like containing them may be used. As the nitrogen source, ammonia gas, aqueous ammonium, ammonium salt and the like can be used. Incidentally, when a nutrient requiring mutant strain for amino acids or the like is used as the host, it is necessary to suitably add the nutrient such as amino acid or the like required by the strain to the medium. An example of the medium for lysine production is shown in Table 1 below as a medium to be used for amino acid production. Incidentally, calcium carbonate is added to other components after being separately sterilized.

Table 1

Medium component	Blending amount
glucose	5 g/dl
$(NH_4)_2SO_4$	2.5 g/dl
KH_2PO_4	0.2 g/dl
$MgSO_4 \cdot 7H_2O$	0.1 g/dl
yeast extract	0.05 g/dl
thiamine hydrochloride	1 μg/l
biotin	300 μg/l
$FeSO_4 \cdot 7H_2O$	1 mg/dl
$MnSO_4 \cdot 4H_2O$	1 mg/dl
calcium carbonate	2.5 g/dl
(pH 7.0)	

The cultivation is performed until generation and accumulation of amino acids substantially stop while suitably controlling pH and temperature of the medium under an aerobic condition. In order to collect amino acids thus accumulated in the cultivated medium, an ordinary method can be applied.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows growth inhibition by 3-bromopyruvate.
Fig. 2 shows growth inhibition by aspartate-β-hydrazide.
Fig. 3 shows growth inhibition by DL-threo-β-hydroxyaspartate.
Fig. 4 shows effects of inhibition recovering substances on 3-bromopyruvate.
Fig. 5 shows effects of inhibition recovering substances on aspartate-β-hydrazide.
Fig. 6 shows effects of inhibition recovering substances on DL-threo-β-hydroxyaspartate.
Fig. 7 shows influences exerted on growth by growth recovering factors.
Fig. 8 shows inhibition of phosphoenolpyruvate carboxylase by growth inhibitory substances.
Fig. 9 shows inhibition of phosphoenolpyruvate carboxylase of the present invention by aspartic acid.
Fig. 10 shows inhibition of phosphoenolpyruvate carboxylase of the present invention by aspartic acid.
Fig. 11 shows a method for introducing mutation into a phosphoenolpyruvate carboxylase gene.
Fig. 12 shows influences exerted by aspartic acid on acitivities of wild type and mutant phosphoenolpyruvate carboxylase in which 438th arginine was substituted with cysteine counted from the N-terminus.
Fig. 13 shows influences exerted by aspartic acid on activities of wild type and mutant phosphoenolpyruvate carboxylase in which 620th lysine was substituted with serine counted from the N-terminus.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will be explained more concretely below with reference to Examples.

Example 1: acquisition of mutant phosphoenolpyruvate carboxylase gene

A mutant gene was prepared by using a plasmid pS2 obtained by inserting a phosphoenolpyruvat carboxylase gene having been cloned and determined for its base sequence into a SalI site of a vector plasmid pBR322. pS2 has an ampicillin resistance gene as a drug resistance marker gene (Sabe, H. et al., Gene, 31, 279-283 (1984)). The nucleotide sequence of the phosphoenolpyruvate carboxylase gene contained in pS2 is the same as that contained in the aforementioned plasmid pT2.

pS2 DNA was treated at 75 °C for 2 hours with a hydroxylamine treating solution (20 μg/ml pS2 DNA, 0.05 M sodium phosphate (pH 6.0), 1 mM EDTA, 0.4 M hydroxylamine). Because of influence by pH on the hydroxylamine treatment, 80 μl of 1 M hydroxylamine • HCl and 1 mM EDTA solution having a pH adjusted to 6.0 with sodium hydroxide, 100 μl of 0.1 M sodium phosphate (pH 6.0) and 1 mM EDTA solution, and TE (10 mM Tris-HCl, 1 mM EDTA) buffer containing 2 μg of pS2 DNA were mixed, to finally provide 200 μl with water.

The aforementioned condition is a condition in which transformants has a survival ratio of 0.2 % based on a state before the treatment in an ampicillin-containing medium when Escherichia coli HB101 is transformed with pS2 after the treatment.

Escherichia coli HB101 was transformed with pS2 treated with hydroxylamine, which was spread on a solid plate medium containing ampicillin to obtain about 10000 colonies of transformants. They were suspended in a liquid medium, and spread on a solid plate medium containing any one of 3-bromopyruvate (3BP), aspartate-β-hydroxamate (AHX), aspartate-β-hydrazide (AHY) and DL-threo-β-hydroxyaspartate (βHA) as the analog compounds of aspartic acid at a concentration near a minimal inhibitory concentration to give 10^3 to 10^5 cells per one medium plate, and growing colonies were selected.

From 100 strains of analog compound resistant strains thus obtained, phosphoenolpyruvate carboxylase produced by each of them was partially purified in accordance with a method described in The Journal of Biochemistry, Vol. 67, No. 4 (1970), and inhibition of enzyme activity by the analog compounds was investigated. Measurement of the enzyme activity was performed in the same manner as described above.

Further, plasmids were isolated from bacterial strains producing mutant enzymes with activities not inhibited by the analog compounds, and were introduced into Escherichia coli PCR1 as a phosphoenolpyruvate carboxylase deficient strain (Sabe, H. et al., Gene, 31, 279-283 (1984)), to confirm production of the mutant enzymes.

Five transformants harboring mutant enzyme genes were thus obtained. As a result of determination of base sequences of these genes, 2 strains had the same mutation, and 4 kinds of mutant genes were obtained. The transformants harboring them were designated as AJ12907, AJ12908, AJ12909 and AJ12910, and were deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan; zip code 305) on August 3, 1993 under the deposition numbers of FERM P-13774, FERM P-13775, FERM P-13776 and FERM P-13777, transferred from the original deposition to international deposition based on Budapest Treaty on July 11, 1994 and has been deposited as deposition numbers of FERM BP-4734、FERM BP-4735、FERM BP-4736、FERM BP-4737, respectively in this order. Further, the plasmids possessed by them were designated as pBP5, pHA19, pBP122 and pR6 respectively in this order. Mutations possessed by the phosphoenolpyruvate carboxylase genes contained in each of the plasmids are shown in Table 2. Numerical values in the table indicate nucleotide numbers or amino acid numbers in SEQ ID NO:1.

Table 2

Transformant	Plasmid	Mutation	Amino acid replacement associated with mutation
AJ12907	pBP5	^{2109}G→A	^{625}Glu→Lys
AJ12908	pHA19	^{901}G→A	^{222}Arg→His
		^{903}G→A	^{223}Glu→Lys
AJ12909	pBP122	^{1099}C→T	^{288}Ser→Phe
		^{1101}G→A	^{289}Glu→Lys
		^{1889}G→A	^{551}Met→Ile
		^{2646}G→A	^{804}Glu→Lys
AJ12910	pR6	^{2835}G→A	^{867}Ala→Thr

Incidentally, selection was performed for AJ12907 and AJ12909 in a medium containing 500 µg/ml of 3BP, for AJ12908 in a medium containing 1000 µg/ml of βHA, and for AJ12910 in a medium containing 500 µg/ml of AHY.

Example 2: mutant phosphoenolpyruvate carboxylase

Sensitivity to aspartic acid was investigated for phosphoenolpyruvate carboxylases produced by the aforementioned 4 transformants. These bacterial strains are deficient in the phosphoenolpyruvate carboxylase gene originating from the host, so that produced phosphoenolpyruvate carboxylase originates from the plasmid.

Sensitivity to aspartic acid was investigated in accordance with a known method (Yoshinaga, T., Izui, K. and Katsuki, H., J. Biochem., 68, 747-750 (1970)). Namely, as a result of measurement of the enzyme activity produced by each of the transformants or Escherichia coli harboring pS2 in the presence of acetyl-coenzyme A known to affect the activity in an activity measurement system at a concentration of 0.1 mM or 1 mM, sensitivity to aspartic acid was measured as shown in Figs. 9 and 10.

According to the result, it is apparent that the wild type enzyme loses its activity when aspartic acid is at a high concentration, while the mutant phosphoenolpyruvate carboxylase of the present invention substantially continues to maintain its activity.

Example 3: fermentative production of L-threonine by Escherichia coli with introduced mutant phosphoenolpyruvate carboxylase

As threonine-producing bacteria of Escherichia coli, B-3996 strain (Japanese Patent Laid-open No. 3-501682 (PCT)) has the highest production ability among those known at present. Thus upon evaluation of the mutant phosphoenolpyruvate carboxylase, B-3996 was used as the host. This B-3996 strain has been deposited in Research Institute for Genetics and Industrial Microorganism Breeding under a registration number of RIA 1867. Further, pBP5 was selected as the mutant phosphoenolpyruvate carboxylase to be evaluated, which was subjected to an experiment.

The plasmid pBP5 having the mutant phosphoenolpyruvate carboxylase was introduced into Escherichia coli B-3996 in accordance with a method of Hanahan (J. Mol. Biol., Vol. 106, p577 (1983)), and a transformant was isolated. As a control, Escherichia coli B-3996 was transformed in the same manner with pS2 as the plasmid to express the wild type phosphoenolpyruvate carboxylase gene.

When Escherichia coli B-3996 and the transformants therefrom were respectively inoculated in a 500 ml of Sakaguchi flask poured with 20 ml of a medium having a composition in Table 3, and cultivated at 37 °C for 40 hours to investigate a production amount of L-threonine, then results shown in Table 4 were obtained. Incidentally, the aforementioned medium was separated into two: glucose and $MgSO_4 \cdot 7H_2O$, and the other components, and adjusted to have a pH of 7.0 with KOH followed by autoclaving at 115 °C for 10 minutes, and then, after mixing them, separately sterilized $CaCO_3$ was added by 30 g/l.

Table 3

Component	Blending amount (g/l)
glucose	40
$(NH_4)_2SO_4$	16
KH_2PO_4	1
$MgSO_4 \cdot 7H_2O$	1
$FeSO_4 \cdot 7H_2O$	0.01
$MnSO_4 \cdot 5H_2O$	0.01
yeast extract (Difco)	2
L-Met	0.5
$CaCO_3$	30

Table 4

Bacterial strain	Threonine production amount (g/l)
Escherichia coli B-3996	15.7
Escherichia coli B-3996/pS2	15.8
Escherichia coli B-3996/pBP5	16.8

As clarified from the result, Escherichia coli B-3996/pBP5 harboring the mutant enzyme expression plasmid having the DNA sequence of the present invention had an improved threonine-producing ability as compared with Escherichia coli B-3996/pS2 harboring the plasmid to express the wild type enzyme.

Example4: fermentative production of L-alutamic acid by Escherichia coli with introduced mutant phosphoenolpyruvate carboxylase

As glutamic acid-producing bacteria of Escherichia coli, Escherichia coli AJ-12628 described in Japanese Patent Laid-open No. 4-11461 has the highest production ability among those known at present. Thus upon evaluation of the mutant phosphoenolpyruvate carboxylase, AJ-12628 was used as the host.

The AJ-12628 strain has been deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under a registration number of FERM BP-385 Further, pBP5 was selected as the mutant phosphoenolpyruvate carboxylase to be evaluated, which was subjected to an experiment.

The plasmid pBP5 having the mutant phosphoenolpyruvate carboxylase was introduced into Escherichia coli AJ-12628 in accordance with a method of Hanahan (J. Mol. Biol., Vol. 106, p577 (1983)), and a transformant was isolated. In the same manner, a transformant of Escherichia coli AJ-12628 with pS2 was isolated.

When Escherichia coli AJ-12628 and the transformants therefrom were respectively inoculated in a 500 ml of Sakaguchi flask poured with 20 ml of a medium having a composition in Table 5, and cultivated at 37 °C for 36 hours to investigate a production amount of L-glutamic acid, then results shown in Table 6 were obtained. Incidentally, the aforementioned medium was separated into two: glucose and $MgSO_4 \cdot 7H_2O$, and the other components, and adjusted to have a pH of 7.0 with KOH followed by autoclaving at 115 °C for 10 minutes, and then, after mixing them, separately sterilized $CaCO_3$ was added by 30 g/l.

Table 5

Component	Blending amount (g/l)
glucose	40
$(NH_4)_2SO_4$	16
KH_2PO_4	1
$MgSO_4 \cdot 7H_2O$	1
$FeSO_4 \cdot 7H_2O$	0.01
$MnSO_4 \cdot 5H_2O$	0.01
yeast extract (Difco)	2
$CaCO_3$	30

Table 6

Bacterial strain	Glutamic acid production amount (g/l)
Escherichia coli AJ-12628	18.0
Escherichia coli AJ-12628/pS2	18.3
Escherichia coli AJ-12628/pBP5	19.6

As clarified from the result, Escherichia coli AJ-12628/pBP5 harboring the mutant enzyme expression plasmid having the DNA sequence of the present invention had an improved glutamate-producing ability as compared with Escherichia coli AJ-12628/pS2 harboring the plasmid to express the wild type enzyme.

Example 5: production of L-lysine by coryneform bacterium with introduced mutant phosphoenolpyruvate carboxylase

In order to introduce and express the mutant gene in a coryneform bacterium, a promoter originating from a bacterium belonging to the genus Brevibacterium was obtained, and was ligated with the mutant gene to prepare an expression type plasmid. Further, it was introduced into a bacterium belonging to the genus Brevibacterium to perform production of L-lysine.

(1) Acquisition of aspartokinase (AK) gene originating from bacterium belonging to the genus Brevibacterium

Chromosomal DNA was prepared according to an ordinary method from a Brevibacterium lactofermentum (Corynebacterium glutamicum) wild strain (ATCC 13869). An AK gene was amplified from the chromosomal DNA by PCR (polymerase chain reaction; see White, T. J. et al., Trends Genet., 5, 185 (1989)). For DNA primers used in the amplification, an oligonucleotide of 23 mer (SEQ ID NO:3) and an oligonucleotide of 21 mer (SEQ ID NO:4) were synthesized to amplify a region of about 1643 bp coding for the AK gene based on a sequence known in Corynebacterium glutamicum (see Molecular Microbiology (1991) 5 (5), 1197-1204, Mol. Gen. Genet. (1990) 224, 317-324).

The synthesis of DNA was performed in accordance with an ordinary phosphoamidite method (see Tetrahedron Letters (1981), 22, 1859) using a DNA synthesizer model 380B produced by Applied Biosystems Co. In the PCR reaction, DNA Thermal Cycler PJ2000 type produced by Takara Shuzo Co., Ltd. was used, and gene amplification was performed by using Taq DNA polymerase in accordance with a method designated by the manufacturer.

An amplified gene fragment of 1643 kb was confirmed by agarose gel electrophoresis, and then the fragment cut out from the gel was purified by an ordinary method, and was cleaved with restriction enzymes NruI (produced by Takara Shuzo Co., Ltd.) and EcoRI (produced by Takara Shuzo Co., Ltd.). pHSG399 (see Takeshita, S. et al.; Gene (1987), 61, 63-74) was used for a cloning vector for the gene fragment. pHSG399 was cleaved with a restriction enzyme SmaI (produced by Takara Shuzo Co., Ltd.) and a restriction enzyme EcoRI, and ligated with the amplified AK gene fragment.

Ligation of DNA was performed by a designated method by using a DNA ligation kit (produced by Takara Shuzo Co., Ltd.). In such a manner, a plasmid was manufactured in which pHSG399 was ligated with the AK gene fragment amplified from Brevibacterium chromosome. The plasmid having the AK gene originating from ATCC 13869 as the wild strain was designated as p399AKY.

(2) Determination of base sequence of AK gene of Brevibacterium lactofermentum

The AK plasmid, p399AKY was prepared, and the base sequence of the AK gene was determined. Determination of the base sequence was performed in accordance with the method of Sanger et al. (F. Sanger et al.: Proc. Natl. Acad. Sci. USA, 74, 5463 (1977) and so forth). Results are shown in SEQ ID NO:5 and SEQ ID NO:7. The DNA fragments have two open reading frames which correspond to α-subunit and β-subunit of AK, respectively. In SEQ ID NO:5 and SEQ ID NO:7, amino acid sequences corresponding to each of the open reading frames are shown together with nucleotide sequences. Further, only the amino acid sequences corresponding to each of the open reading frames are shown in SEQ ID NO:6 and SEQ ID NO:8.

(3) Preparation of phosphoenolpyruvate carboxylase expression plasmid

SalI fragments of about 4.4 kb containing phosphoenolpyruvate carboxylase genes were extracted from pS2 as the plasmid having the wild type phosphoenolpyruvate carboxylase gene and pBP5 as the plasmid having the obtained

mutant phosphoenolpyruvate carboxylase gene, and inserted into a SalI site of a plasmid vector pHSG399 universally used for Escherichia coli. Manufactured plasmids were designated as pHS2 for the wild type and as pHBP5 for the mutant.

In order to convert pHS2 and pHPB5 into plasmids to express in Brevibacterium, a promoter and a replication origin of a plasmid for functioning in Brevibacterium were introduced. As the promoter, a gene fragment containing one from 1st NruI site to 207th ApaLI site of the base sequence, which was postulated to be a promoter region of the cloned AK gene, was extracted from p399AKY, and inserted into an AvaI site located about 60 bp before the structural genes of pHS2 and pHBP5 to allow the transcription direction to be in a regular direction.

Further, a gene fragment to enable autonomously replication of the plasmid in Brevibacterium, namely the replication origin of the plasmid was introduced into a site located on the vector. A gene fragment containing the replication origin of the plasmid was extracted from a vector pHC4 for Brevibacterium (see paragraph No. 10 in Japanese Patent Laid-open No. 5-7491; Escherichia coli AJ12039 harboring the same plasmid is deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology, to which a deposition number of FERM P12215 is given), and restriction enzyme sites at both termini were modified into PstI sites by introduction of linkers.

This fragment was introduced into a PstI site in a vector portion of the plasmid added with the promoter derived from Brevibacterium. Constructed phosphoenolpyruvate carboxylase-expressing plasmids were designated as pHS2B for a wild type phosphoenolpyruvate carboxylase plasmid originating from pS2 and as pHBP5B for a mutant phosphoenolpyruvate carboxylase plasmid originating from pBP5, respectively.

(4) Production of L-lysine by using phosphoenolpyruvate carboxylase expression type plasmid

Prepared pHS2B and pHBP5B were respectively introduced into AJ3463 as an L-lysine-producing bacterium of Brevibacterium lactofermentum (see Japanese Patent Publication No. 51-34477). For introduction of the gene, a transformation method employing electric pulse was used (see Japanese Patent Laid-open No. 2-207791). The host strain and transformants were cultivated with shaking for 72 hours at 31.5 °C in a lysine production medium having a composition in Table 7. The aforementioned medium was prepared such that those except for $CaCO_3$ among the components listed in the table were added to 1 l of water, and adjusted to have a pH of 8.0 with KOH followed by autoclaving at 115 °C for 15 minutes, and then $CaCO_3$ having been subjected to heat sterilization was further added. Accumulated amounts of L-lysine in the medium after cultivation are shown in Table 8.

Table 7

Component	Blending amount in 1 L
glucose	100 g
$(NH_4)_2SO_4$	55 g
soybean concentrate*	35 ml
KH_2PO_4	1 g
$MgSO_4 \cdot 7H_2O$	1 g
vitamin B1	20 g
biotin	5 g
nicotinic acid amide	5 mg
$FeSO_4 \cdot 7H_2O$	0.01 g
$MnSO_4 \cdot 5H_2O$	0.01 g
$CaCO_3$	50g

*: product of Ajinomoto Co., Ltd. (trade name: Mamenou)

Table 8

Bacterial strain	Lysine production amount (g/l)
Brevibacterium lactof rmentum AJ3463	20.0
Brevibacterium lactofermentum AJ3463/pHS2B	22.0
Brevibacterium lactofermentum AJ3463/pHBP5B	25.0

As shown in the result, Brevibacterium lactofermentum AJ3463/pHBP5B harboring the mutant enzyme expression plasmid having the DNA sequence of the present invention had an improved lysine-producing ability as compared with Brevibacterium lactofermentum AJ3463/pHS2B harboring the plasmid to express the wild type enzyme.

Example 6: another example of mutant phosphoenolpyruvate carboxylase of the present invention and its gene

(1) Preparation of mutant phosphoenolpyruvate carboxylase gene

Upon preparation of DNA coding for a mutant phosphoenolpyruvate carboxylase, a phosphoenolpyruvate carboxylase gene cloned in a plasmid pT2 was used as a material.

A host, which is allowed to harbor the plasmid pT2, is preferably deficient in phosphoenolpyruvate carboxylase gene in order to detect only the activity of phosphoenolpyruvate carboxylase originating from the plasmid. Escherichia coli F15 (Hfr, recA1, met, Δ(ppc-argECBH), Tn10) was used as such a deficient strain. Escherichia coli AJ-12873, which is allowed to harbor pT2 in F15 strain, is deposited as FERM P-13752 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan; zip code 305) on July 15, 1993, transferred from the original deposition to international deposition based on Budapest Treaty on July 11, 1994 and has been deposited as deposition number of FERM BP-4732. In addition, an entire base sequence of pT2 is shown in SEQUENCE ID NO:1.

In order to replace a codon of 438th arginine of the phosphoenolpyruvate carboxylase into a codon of cysteine by using pT2, the Overlapping Extension method (Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R., Gene, 77, 51-59 (1989)) utilizing the PCR (Polymerase Chain Reaction) method was used.

Incidentally, the PCR method is a method in which an amplification cycle comprising thermal denaturation of double strand DNA into single strand DNA, annealing of oligonucleotide primers corresponding to sequences at both ends of a site aimed to be amplified and the aforementioned thermally denatured DNA, and polymerase reaction using the aforementioned oligonucleotides as primers is repeated, thereby the aforementioned DNA sequence is amplified in a manner of an exponential function.

A region subjected to site specific mutation by the PCR method is shown in Fig. 11. The primers used in the present invention were 4 species of a primer c (SEQUENCE ID NO:11, corresponding to base Nos. 1535-1554 in SEQUENCE ID NO:1) having a sequence in the vicinity of the codon of 438th arginine, a primer b (SEQUENCE ID NO:10) having a sequence complement to the primer c, a primer a (SEQUENCE ID NO:9, corresponding to base Nos. 1185-1200 in SEQUENCE ID NO:1) having a sequence upstream therefrom, and a primer d (SEQUENCE ID NO:12, corresponding to base Nos. 2327-2342 in SEQUENCE ID NO:1) having a sequence complement to a downstream sequence.

In the primer b and the primer c, the codon (CGT) of 438th arginine was replaced with a codon (TGT) of cysteine. This replacement may use TGC which is another codon of cysteine. Further, C of the third letter of a codon (AAC) of 435th asparagine was replaced with T, and hence an EcoRI site was internally introduced with no replacement of amino acid, so that a mutant plasmid could be selected by using it as an index. However, this mutation is not essential to the present invention.

When the PCR reaction was performed by using pT2 DNA as a template and the primer a and the primer b as the primers, a fragment from the upstream of the mutation site to the mutation site (AB fragment in Fig. 11) was amplified. Further, when the PCR reaction was performed by using the primer c and the primer d, a fragment downstream from the mutation site (CD fragment in Fig. 11) was amplified. When each of the amplified products (AB, CD) was annealed again after thermal denaturation to perform a polymerase reaction, they were ligated to obtain a fragment (AD fragment in Fig. 11). Incidentally, the PCR reaction was performed by repeating 30 cycles of each comprising heating at 94 °C for 1 minute followed by denaturation (94 °C, 1.5 minutes), annealing (50 °C, 2 minutes), and elongation reaction by polymerase (72 °C, 3.5 minutes). In addition, reaction compositions are shown in Table 9.

Table 9

Composition (( ): final conc.)	PCR fragment		
	AB	CD	AD
H_2O	53.5	53.5	53.5
10-fold reaction buffer	10	10	10
mixture of 1.25 mM dNTP	16	16	16
20 μM primer a (1 μM)	5	-	5
20 μM primer b (1 μM)	5	-	-
20 μM primer c (1 μM)	-	5	-
20 μM primer d (1 μM)	-	5	5
10 μg/μl pT2 (0.1 μg)	10	10	-
PCR fragment AB*	-	-	5
PCR fragment CD*	-	-	5
2.5 U/μl Taq polymerase	0.5	0.5	0.5
total amount	100 μl	100 μl	100 μl

*: PCR fragments AB and CD were prepared, after the PCR reaction, by recovering 10 μl thereof from polyacrylamide gel, and dissolving it in 5 μl of TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)).

In the AD fragment obtained as described above, a BssHII site (1231-1236 in SEQ ID NO:1) at the upstream side and a SpII site (2249-2254 in SEQ ID NO:1) at the downstream side were present, so that complete digestion was performed with these enzymes to make replacement for a corresponding region of the plasmid pT2 (Fig. 11).

(2) Selection of inhibition-desensitized phosphoenolpyruvate carboxylase

Escherichia coli was transformed with a plasmid obtained as described above, and a transformed strain was cultivated to recover the plasmid to select one cleaved by EcoRI. With respect to selected DNA, a base sequence of the region amplified by the aforementioned PCR method was determined by the dideoxy method to confirm that base replacement as exactly aimed was introduced. This plasmid was designated as pT2R438C. A strain (AJ12874) obtained by introducing this plasmid into the aforementioned Escherichia coli F15 has been deposited as FERM P-13753 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan; zip code 305) on July 15, 1993, transferred from the original deposition to international deposition based on Budapest Treaty on July 11, 1994 and has been deposited as deposition number of FERM BP-4733.

The base sequence of pT2R438C is a sequence in which 1541th and 1550th nucleotides are replaced from C to T respectively in SEQ ID NO:1.

(3) Confirmation of desensitization of inhibition of mutant phosphoenolpyruvate carboxylase by aspartic acid

Sensitivity to aspartic acid was investigated for phosphoenolpyruvate carboxylase produced by the aforementioned Escherichia coli AJ12874 harboring pT2R438C. Incidentally, as described above, because the Escherichia coli F15 is deficient in phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxylase produced by AJ12874 originates from the plasmid.

Sensitivity to aspartic acid was investigated in accordance with a known method (Yoshinaga, T., Izui, K. and Katsuki, H., J. Biochem., 68, 747-750 (1970)). Namely, as a result of measurement of the enzyme activity in the presence of acetyl-coenzyme A known to affect the activity in an activity measurement system at a concentration of 1 mM or 2 mM, sensitivity to aspartic acid was measured as shown in Fig. 12.

It is apparent that the wild type enzyme substantially loses its activity when aspartic acid is at a high concentration, while the mutant phosphoenolpyruvate carboxylase of the present invention continues to maintain its activity.

(4) Preparation of mutant phosphoenolpyruvate carboxylase gene (II)

In order to replace a codon of 620th lysine with a codon of serine in the phosphoenolpyruvate carboxylase gene carried on the plasmid pT2, the Overlapping Extension method (Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R., Gene, 77, 5l-59 (l989)) utilizing the PCR (Polymerase Chain Reaction) method was used. Concrete procedures were in accordance with the method described in (1). A plasmid carrying a mutant gene constructed with aimed replacement was designated as pT2K620S. Further, an obtained mutant enzyme was designated as K620S mutant enzyme.

(5) Confirmation of desensitization of inhibition by aspartic acid concerning mutant phosphoenolpyruvate carboxylase.

With respect to the phosphoenolpyruvic carboxylase produced by a transformant obtained by introducing the plasmid pT2K620S into the aforementioned Escherichia coli Fl5, sensitivity to aspartic acid was investigated. Incidentally, as described above, since the Escherichia coli Fl5 lacks phosphoenolpyruvate carboxylase, any phosphoenolpyruvate carboxylase produced by the transformant originates from the plasmid.

Sensitivity to aspartic acid was investigated in accordance with a known method (Yoshinaga, T., Izui, K. and Katsuki, H., J. Biochem., 68, 747-750 (l970)). Namely, as a result of measurement of the enzyme activity in the presence of acetyl-coenzyme A known to affect the activity in an activity measurement system at a concentration of l mM or 2 mM, sensitivity to aspartic acid was measured as shown in Fig. 13.

It is apparent that the wild enzyme substantially loses its activity when aspartic acid is at a high concentration, while the type phosphoenolpyruvate carboxylase of the present invention continues to maintain its activity.

In Fig. l3, sensitivity to aspartic acid is also depicted for a mutant phosphoenolpyruvate carboxylase in which 650th lysine is replaced with serine (K650A mutant enzyme), and for a mutant phosphoenolpyruvate carboxylase in which 491th lysine is replaced with serine (K491A mutant enzyme). In the case of these mutant enzymes, inhibition by aspartic acid was not desensitized.

INDUSTRIAL APPLICABILITY

The DNA sequence of the present invention codes for the mutant phosphoenolpyruvate carboxylase, and the microorganism harboring this DNA sequence produces the aforementioned enzyme.

The mutant phosphoenolpyruvate carboxylase of the present invention does not substantially undergo activity inhibition by aspartic acid, so that it can be utilized for fermentative production of amino acids subjected to regulation of biosynthesis by aspartic acid and the like.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: Ajinomoto Co. Inc.
(A) NAME:
(B) STREET: 15-1, Kyobashi 1-chome, Chuo-ku
(C) CITY: Tokyo
(D) STATE OR PROVINCE:
(E) COUNTRY: Japan
(F) POSTAL CODE: 104

(ii) TITLE OF INVENTION: Mutant Phosphoenolpyruvate Carboxylase, Its gene, and Production Method of Amino Acid
(iii) NUMBER OF SEQUENCES:12
(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5186
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular
(ii) MOLECULAR TYPE: other..genomic DNA and vector DNA
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 237..2888
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCGACCGGCG ATTTTTTAAC ATTTCCATAA GTTACGCTTA TTTAAAGCGT CGTGAATTTA 60
ATGACGTAAA TTCCTGCTAT TTATTCGTTT GCTGAAGCGA TTTCGCAGCA TTTGACGTCA 120
CCGCTTTTAC GTGGCTTTAT AAAAGACGAC GAAAAGCAAA GCCCGAGCAT ATTCGCGCCA 180

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ATGCGACGTG AAGGATACAG GCTATCAAA CGATAAGATG GGGTGTCTGG GGTAAT          236
ATG AAC GAA CAA TAT TCC GCA TTG CGT AGT AAT GTC AGT ATG CTC GGC        284
Met Asn Glu Gln Tyr Ser Ala Leu Arg Ser Asn Val Ser Met Leu Gly
 1               5                  10                  15
AAA GTG CTG GGA GAA ACC ATC AAG GAT GCG TTG GGA GAA CAC ATT CTT        332
Lys Val Leu Gly Glu Thr Ile Lys Asp Ala Leu Gly Glu His Ile Leu
            20                  25                  30
GAA CGC GTA GAA ACT ATC CGT AAG TTG TCG AAA TCT TCA CGC GCT GGC        380
Glu Arg Val Glu Thr Ile Arg Lys Leu Ser Lys Ser Ser Arg Ala Gly
        35                  40                  45
AAT GAT GCT AAC CGC CAG GAG TTG CTC ACC ACC TTA CAA AAT TTG TCG        428
Asn Asp Ala Asn Arg Gln Glu Leu Leu Thr Thr Leu Gln Asn Leu Ser
    50                  55                  60
AAC GAC GAG CTG CTG CCC GTT GCG CGT GCG TTT AGT CAG TTC CTG AAC        476
Asn Asp Glu Leu Leu Pro Val Ala Arg Ala Phe Ser Gln Phe Leu Asn
65                  70                  75                  80
CTG GCC AAC ACC GCC GAG CAA TAC CAC AGC ATT TCG CCG AAA GGC GAA        524
Leu Ala Asn Thr Ala Glu Gln Tyr His Ser Ile Ser Pro Lys Gly Glu
                85                  90                  95
GCT GCC AGC AAC CCG GAA GTG ATC GCC CGC ACC CTG CGT AAA CTG AAA        572
Ala Ala Ser Asn Pro Glu Val Ile Ala Arg Thr Leu Arg Lys Leu Lys
            100                 105                 110
AAC CAG CCG GAA CTG AGC GAA GAC ACC ATC AAA AAA GCA GTG GAA TCG        620
Asn Gln Pro Glu Leu Ser Glu Asp Thr Ile Lys Lys Ala Val Glu Ser
        115                 120                 125
CTG TCG CTG GAA CTG GTC CTC ACG GCT CAC CCA ACC GAA ATT ACC CGT        668
Leu Ser Leu Glu Leu Val Leu Thr Ala His Pro Thr Glu Ile Thr Arg
    130                 135                 140
CGT ACA CTG ATC CAC AAA ATG GTG GAA GTG AAC GCC TGT TTA AAA CAG        716
Arg Thr Leu Ile His Lys Met Val Glu Val Asn Ala Cys Leu Lys Gln
145                 150                 155                 160
CTC GAT AAC AAA GAT ATC GCT GAC TAC GAA CAC AAC CAG CTG ATG CGT        764
Leu Asp Asn Lys Asp Ile Ala Asp Tyr Glu His Asn Gln Leu Met Arg
                165                 170                 175
CGC CTG CGC CAG TTG ATC GCC CAG TCA TGG CAT ACC GAT GAA ATC CGT        812
Arg Leu Arg Gln Leu Ile Ala Gln Ser Trp His Thr Asp Glu Ile Arg
            180                 185                 190
AAG CTG CGT CCA AGC CCG GTA GAT GAA GCC AAA TGG GGC TTT GCC GTA        860
Lys Leu Arg Pro Ser Pro Val Asp Glu Ala Lys Trp Gly Phe Ala Val
        195                 200                 205
GTG GAA AAC AGC CTG TGG CAA GGC GTA CCA AAT TAC CTG CGC GAA CTG        908
Val Glu Asn Ser Leu Trp Gln Gly Val Pro Asn Tyr Leu Arg Glu Leu
    210                 215                 220
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AAC GAA CAA CTG GAA GAG AAC CTC GGC TAC AAA CTG CCC GTC GAA TTT      956
Asn Glu Gln Leu Glu Glu Asn Leu Gly Tyr Lys Leu Pro Val Glu Phe
225                 230                 235                 240
GTT CCG GTC CGT TTT ACT TCG TGG ATG GGC GGC GAC CGC GAC GGC AAC     1004
Val Pro Val Arg Phe Thr Ser Trp Met Gly Gly Asp Arg Asp Gly Asn
                245                 250                 255
CCG AAC GTC ACT GCC GAT ATC ACC CGC CAC GTC CTG CTA CTC AGC CGC     1052
Pro Asn Val Thr Ala Asp Ile Thr Arg His Val Leu Leu Leu Ser Arg
            260                 265                 270
TGG AAA GCC ACC GAT TTG TTC CTG AAA GAT ATT CAG GTG CTG GTT TCT     1100
Trp Lys Ala Thr Asp Leu Phe Leu Lys Asp Ile Gln Val Leu Val Ser
        275                 280                 285
GAA CTG TCG ATG GTT GAA GCG ACC CCT GAA CTG CTG GCG CTG GTT GGC     1148
Glu Leu Ser Met Val Glu Ala Thr Pro Glu Leu Leu Ala Leu Val Gly
    290                 295                 300
GAA GAA GGT GCC GCA GAA CCG TAT CGC TAT CTG ATG AAA AAC CTG CGT     1196
Glu Glu Gly Ala Ala Glu Pro Tyr Arg Tyr Leu Met Lys Asn Leu Arg
305                 310                 315                 320
TCT CGC CTG ATG GCG ACA CAG GCA TGG CTG GAA GCG CGC CTG AAA GGC     1244
Ser Arg Leu Met Ala Thr Gln Ala Trp Leu Glu Ala Arg Leu Lys Gly
                325                 330                 335
GAA GAA CTG CCA AAA CCA GAA GGC CTG CTG ACA CAA AAC GAA GAA CTG     1292
Glu Glu Leu Pro Lys Pro Glu Gly Leu Leu Thr Gln Asn Glu Glu Leu
            340                 345                 350
TGG GAA CCG CTC TAC GCT TGC TAC CAG TCA CTT CAG GCG TGT GGC ATG     1340
Trp Glu Pro Leu Tyr Ala Cys Tyr Gln Ser Leu Gln Ala Cys Gly Met
        355                 360                 365
GGT ATT ATC GCC AAC GGC GAT CTG CTC GAC ACC CTG CGC CGC GTG AAA     1388
Gly Ile Ile Ala Asn Gly Asp Leu Leu Asp Thr Leu Arg Arg Val Lys
    370                 375                 380
TGT TTC GGC GTA CCG CTG GTC CGT ATT GAT ATC CGT CAG GAG AGC ACG     1436
Cys Phe Gly Val Pro Leu Val Arg Ile Asp Ile Arg Gln Glu Ser Thr
385                 390                 395                 400
CGT CAT ACC GAA GCG CTG GGC GAG CTG ACC CGC TAC CTC GGT ATC GGC     1484
Arg His Thr Glu Ala Leu Gly Glu Leu Thr Arg Tyr Leu Gly Ile Gly
                405                 410                 415
GAC TAC GAA AGC TGG TCA GAG GCC GAC AAA CAG GCG TTC CTG ATC CGC     1532
Asp Tyr Glu Ser Trp Ser Glu Ala Asp Lys Gln Ala Phe Leu Ile Arg
            420                 425                 430
GAA CTG AAC TCC AAA CGT CCG CTT CTG CCG CGC AAC TGG CAA CCA AGC     1580
Glu Leu Asn Ser Lys Arg Pro Leu Leu Pro Arg Asn Trp Gln Pro Ser
        435                 440                 445
GCC GAA ACG CGC GAA GTG CTC GAT ACC TGC CAG GTG ATT GCC GAA GCA     1628
Ala Glu Thr Arg Glu Val Leu Asp Thr Cys Gln Val Ile Ala Glu Ala
    450                 455                 460
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CCG CAA GGC TCC ATT GCC GCC TAC GTG ATC TCG ATG GCG AAA ACG CCG      1676
Pro Gln Gly Ser Ile Ala Ala Tyr Val Ile Ser Met Ala Lys Thr Pro
465                 470                 475                 480
TCC GAC GTA CTG GCT GTC CAC CTG CTG CTG AAA GAA GCG GGT ATC GGG      1724
Ser Asp Val Leu Ala Val His Leu Leu Leu Lys Glu Ala Gly Ile Gly
                485                 490                 495
TTT GCG ATG CCG GTT GCT CCG CTG TTT GAA ACC CTC GAT GAT CTG AAC      1772
Phe Ala Met Pro Val Ala Pro Leu Phe Glu Thr Leu Asp Asp Leu Asn
            500                 505                 510
AAC GCC AAC GAT GTC ATG ACC CAG CTG CTC AAT ATT GAC TGG TAT CGT      1820
Asn Ala Asn Asp Val Met Thr Gln Leu Leu Asn Ile Asp Trp Tyr Arg
        515                 520                 525
GGC CTG ATT CAG GGC AAA CAG ATG GTG ATG ATT GGC TAT TCC GAC TCA      1868
Gly Leu Ile Gln Gly Lys Gln Met Val Met Ile Gly Tyr Ser Asp Ser
    530                 535                 540
GCA AAA GAT GCG GGA GTG ATG GCA GCT TCC TGG GCG CAA TAT CAG GCA      1916
Ala Lys Asp Ala Gly Val Met Ala Ala Ser Trp Ala Gln Tyr Gln Ala
545                 550                 555                 560
CAG GAT GCA TTA ATC AAA ACC TGC GAA AAA GCG GGT ATT GAG CTG ACG      1964
Gln Asp Ala Leu Ile Lys Thr Cys Glu Lys Ala Gly Ile Glu Leu Thr
                565                 570                 575
TTG TTC CAC GGT CGC GGC GGT TCC ATT GGT CGC GGC GGC GCA CCT GCT      2012
Leu Phe His Gly Arg Gly Gly Ser Ile Gly Arg Gly Gly Ala Pro Ala
            580                 585                 590
CAT GCG GCG CTG CTG TCA CAA CCG CCA GGA AGC CTG AAA GGC GGC CTG      2060
His Ala Ala Leu Leu Ser Gln Pro Pro Gly Ser Leu Lys Gly Gly Leu
        595                 600                 605
CGC GTA ACC GAA CAG GGC GAG ATG ATC CGC TTT AAA TAT GGT CTG CCA      2108
Arg Val Thr Glu Gln Gly Glu Met Ile Arg Phe Lys Tyr Gly Leu Pro
    610                 615                 620
GAA ATC ACC GTC AGC AGC CTG TCG CTT TAT ACC GGG GCG ATT CTG GAA      2156
Glu Ile Thr Val Ser Ser Leu Ser Leu Tyr Thr Gly Ala Ile Leu Glu
625                 630                 635                 640
GCC AAC CTG CTG CCA CCG CCG GAG CCG AAA GAG AGC TGG CGT CGC ATT      2204
Ala Asn Leu Leu Pro Pro Pro Glu Pro Lys Glu Ser Trp Arg Arg Ile
                645                 650                 655
ATG GAT GAA CTG TCA GTC ATC TCC TGC GAT GTC TAC CGC GGC TAC GTA      2252
Met Asp Glu Leu Ser Val Ile Ser Cys Asp Val Tyr Arg Gly Tyr Val
            660                 665                 670
CGT GAA AAC AAA GAT TTT GTG CCT TAC TTC CGC TCC GCT ACG CCG GAA      2300
Arg Glu Asn Lys Asp Phe Val Pro Tyr Phe Arg Ser Ala Thr Pro Glu
        675                 680                 685
CAA GAA CTG GGC AAA CTG CCG TTG GGT TCA CGT CCG GCG AAA CGT CGC      2348
Gln Glu Leu Gly Lys Leu Pro Leu Gly Ser Arg Pro Ala Lys Arg Arg
    690                 695                 700
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CCA ACC GGC GGC GTC GAG TCA CTA CGC GCC ATT CCG TGG ATC TTC GCC     2396
Pro Thr Gly Gly Val Glu Ser Leu Arg Ala Ile Pro Trp Ile Phe Ala
705                 710                 715                 720
TGG ACG CAA AAC CGT CTG ATG CTC CCC GCC TGG CTG GGT GCA GGT ACG     2444
Trp Thr Gln Asn Arg Leu Met Leu Pro Ala Trp Leu Gly Ala Gly Thr
                725                 730                 735
GCG CTG CAA AAA GTG GTC GAA GAC GGC AAA CAG AGC GAG CTG GAG GCT     2492
Ala Leu Gln Lys Val Val Glu Asp Gly Lys Gln Ser Glu Leu Glu Ala
            740                 745                 750
ATG TGC CGC GAT TGG CCA TTC TTC TCG ACG CGT CTC GGC ATG CTG GAG     2540
Met Cys Arg Asp Trp Pro Phe Phe Ser Thr Arg Leu Gly Met Leu Glu
        755                 760                 765
ATG GTC TTC GCC AAA GCA GAC CTG TGG CTG GCG GAA TAC TAT GAC CAA     2588
Met Val Phe Ala Lys Ala Asp Leu Trp Leu Ala Glu Tyr Tyr Asp Gln
    770                 775                 780
CGC CTG GTA GAC AAA GCA CTG TGG CCG TTA GGT AAA GAG TTA CGC AAC     2636
Arg Leu Val Asp Lys Ala Leu Trp Pro Leu Gly Lys Glu Leu Arg Asn
785                 790                 795                 800
CTG CAA GAA GAA GAC ATC AAA GTG GTG CTG GCG ATT GCC AAC GAT TCC     2684
Leu Gln Glu Glu Asp Ile Lys Val Val Leu Ala Ile Ala Asn Asp Ser
                805                 810                 815
CAT CTG ATG GCC GAT CTG CCG TGG ATT GCA GAG TCT ATT CAG CTA CGG     2732
His Leu Met Ala Asp Leu Pro Trp Ile Ala Glu Ser Ile Gln Leu Arg
            820                 825                 830
AAT ATT TAC ACC GAC CCG CTG AAC GTA TTG CAG GCC GAG TTG CTG CAC     2780
Asn Ile Tyr Thr Asp Pro Leu Asn Val Leu Gln Ala Glu Leu Leu His
        835                 840                 845
CGC TCC CGC CAG GCA GAA AAA GAA GGC CAG GAA CCG GAT CCT CGC GTC     2828
Arg Ser Arg Gln Ala Glu Lys Glu Gly Gln Glu Pro Asp Pro Arg Val
    850                 855                 860
GAA CAA GCG TTA ATG GTC ACT ATT GCC GGG ATT GCG GCA GGT ATG CGT     2876
Glu Gln Ala Leu Met Val Thr Ile Ala Gly Ile Ala Ala Gly Met Arg
865                 870                 875                 880
AAT ACC GGC TAATCTTCCT CTTCTGCAAA CCCTCGTGCT TTTGCGCGAG             2925
Asn Thr Gly

GGTTTTCTGA AATACTTCTG TTCTAACACC CTCGTTTTCA ATATATTTCT GTCTGCATTT   2985
TATTCAAATT CTGAATATAC CTTCAGATAT CCTTAAGGGC CTCGTGATAC GCCTATTTTT   3045
ATAGGTTAAT GTCATGATAA TAATGGTTTC TTAGACGTCA GGTGGCACTT TTCGGGGAAA   3105
TGTGCGCGGA ACCCCTATTT GTTTATTTTT CTAAATACAT TCAAATATGT ATCCGCTCAT   3165
GAGACAATAA CCCTGATAAA TGCTTCAATA ATATTGAAAA AGGAAGAGTA TGAGTATTCA   3225
ACATTTCCGT GTCGCCCTTA TTCCCTTTTT TGCGGCATTT TGCCTTCCTG TTTTTGCTCA   3285
CCCAGAAACG CTGGTGAAAG TAAAAGATGC TGAAGATCAG TTGGGTGCAC GAGTGGGTTA   3345
CATCGAACTG GATCTCAACA GCGGTAAGAT CCTTGAGAGT TTTCGCCCCG AAGAACGTTT   3405
TCCAATGATG AGCACTTTTA AAGTTCTGCT ATGTGGCGCG GTATTATCCC GTATTGACGC   3465
CGGGCAAGAG CAACTCGGTC GCCGCATACA CTATTCTCAG AATGACTTGG TTGAGTACTC   3525
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ACCAGTCACA GAAAAGCATC TTACGGATGG CATGACAGTA AGAGAATTAT GCAGTGCTGC   3585
CATAACCATG AGTGATAACA CTGCGGCCAA CTTACTTCTG ACAACGATCG GAGGACCGAA   3645
GGAGCTAACC GCTTTTTTGC ACAACATGGG GGATCATGTA ACTCGCCTTG ATCGTTGGGA   3705
ACCGGAGCTG AATGAAGCCA TACCAAACGA CGAGCGTGAC ACCACGATGC CTGTAGCAAT   3765
GGCAACAACG TTGCGCAAAC TATTAACTGG CGAACTACTT ACTCTAGCTT CCCGGCAACA   3825
ATTAATAGAC TGGATGGAGG CGGATAAAGT TGCAGGACCA CTTCTGCGCT CGGCCCTTCC   3885
GGCTGGCTGG TTTATTGCTG ATAAATCTGG AGCCGGTGAG CGTGGGTCTC GCGGTATCAT   3945
TGCAGCACTG GGGCCAGATG GTAAGCCCTC CCGTATCGTA GTTATCTACA CGACGGGGAG   4005
TCAGGCAACT ATGGATGAAC GAAATAGACA GATCGCTGAG ATAGGTGCCT CACTGATTAA   4065
GCATTGGTAA CTGTCAGACC AAGTTTACTC ATATATACTT TAGATTGATT TAAAACTTCA   4125
TTTTTAATTT AAAAGGATCT AGGTGAAGAT CCTTTTTGAT AATCTCATGA CCAAAATCCC   4185
TTAACGTGAG TTTTCGTTCC ACTGAGCGTC AGACCCCGTA GAAAAGATCA AAGGATCTTC   4245
TTGAGATCCT TTTTTTCTGC GCGTAATCTG CTGCTTGCAA ACAAAAAAAC CACCGCTACC   4305
AGCGGTGGTT TGTTTGCCGG ATCAAGAGCT ACCAACTCTT TTTCCGAAGG TAACTGGCTT   4365
CAGCAGAGCG CAGATACCAA ATACTGTCCT TCTAGTGTAG CCGTAGTTAG GCCACCACTT   4425
CAAGAACTCT GTAGCACCGC CTACATACCT CGCTCTGCTA ATCCTGTTAC CAGTGGCTGC   4485
TGCCAGTGGC GATAAGTCGT GTCTTACCGG GTTGGACTCA AGACGATAGT TACCGGATAA   4545
GGCGCAGCGG TCGGGCTGAA CGGGGGGTTC GTGCACACAG CCCAGCTTGG AGCGAACGAC   4605
CTACACCGAA CTGAGATACC TACAGCGTGA GCATTGAGAA AGCGCCACGC TTCCCGAAGG   4665
GAGAAAGGCG GACAGGTATC CGGTAAGCGG CAGGGTCGGA ACAGGAGAGC GCACGAGGGA   4725
GCTTCCAGGG GGAAACGCCT GGTATCTTTA TAGTCCTGTC GGGTTTCGCC ACCTCTGACT   4785
TGAGCGTCGA TTTTTGTGAT GCTCGTCAGG GGGGCGGAGC CTATGGAAAA ACGCCAGCAA   4845
CGCGGCCTTT TTACGGTTCC TGGCCTTTTG CTGGCCTTTT GCTCACATGT TCTTTCCTGC   4905
GTTATCCCCT GATTCTGTGG ATAACCGTAT TACCGCCTTT GAGTGAGCTG ATACCGCTCG   4965
CCGCAGCCGA ACGACCGAGC GCAGCGAGTC AGTGAGCGAG GAAGCGGAAG AGCGCCCAAT   5025
ACGCAAACCG CCTCTCCCCG CGCGTTGGCC GATTCATTAA TGCAGAAGGG TTGGTTTGCG   5085
CATTCACAGT TCTCCGCAAG AATTGATTGG CTCCAATTCT TGGAGTGGTG AATCCGTTAG   5145
CGAGGTGCCG CCGGCTTCCA TTCAGGTCGA GGTGGCCCGG G                       5186
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 883 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Asn Glu Gln Tyr Ser Ala Leu Arg Ser Asn Val Ser Met Leu Gly
 1               5                  10                  15
Lys Val Leu Gly Glu Thr Ile Lys Asp Ala Leu Gly Glu His Ile Leu
            20                  25                  30
Glu Arg Val Glu Thr Ile Arg Lys Leu Ser Lys Ser Ser Arg Ala Gly
        35                  40                  45
Asn Asp Ala Asn Arg Gln Glu Leu Leu Thr Thr Leu Gln Asn Leu Ser
    50                  55                  60
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Asn Asp Glu Leu Leu Pro Val Ala Arg Ala Phe Ser Gln Phe Leu Asn
 65                  70                  75                  80
Leu Ala Asn Thr Ala Glu Gln Tyr His Ser Ile Ser Pro Lys Gly Glu
                 85                  90                  95
Ala Ala Ser Asn Pro Glu Val Ile Ala Arg Thr Leu Arg Lys Leu Lys
            100                 105                 110
Asn Gln Pro Glu Leu Ser Glu Asp Thr Ile Lys Lys Ala Val Glu Ser
        115                 120                 125
Leu Ser Leu Glu Leu Val Leu Thr Ala His Pro Thr Glu Ile Thr Arg
    130                 135                 140
Arg Thr Leu Ile His Lys Met Val Glu Val Asn Ala Cys Leu Lys Gln
145                 150                 155                 160
Leu Asp Asn Lys Asp Ile Ala Asp Tyr Glu His Asn Gln Leu Met Arg
                165                 170                 175
Arg Leu Arg Gln Leu Ile Ala Gln Ser Trp His Thr Asp Glu Ile Arg
            180                 185                 190
Lys Leu Arg Pro Ser Pro Val Asp Glu Ala Lys Trp Gly Phe Ala Val
        195                 200                 205
Val Glu Asn Ser Leu Trp Gln Gly Val Pro Asn Tyr Leu Arg Glu Leu
    210                 215                 220
Asn Glu Gln Leu Glu Glu Asn Leu Gly Tyr Lys Leu Pro Val Glu Phe
225                 230                 235                 240
Val Pro Val Arg Phe Thr Ser Trp Met Gly Gly Asp Arg Asp Gly Asn
                245                 250                 255
Pro Asn Val Thr Ala Asp Ile Thr Arg His Val Leu Leu Leu Ser Arg
            260                 265                 270
Trp Lys Ala Thr Asp Leu Phe Leu Lys Asp Ile Gln Val Leu Val Ser
        275                 280                 285
Glu Leu Ser Met Val Glu Ala Thr Pro Glu Leu Leu Ala Leu Val Gly
    290                 295                 300
Glu Glu Gly Ala Ala Glu Pro Tyr Arg Tyr Leu Met Lys Asn Leu Arg
305                 310                 315                 320
Ser Arg Leu Met Ala Thr Gln Ala Trp Leu Glu Ala Arg Leu Lys Gly
                325                 330                 335
Glu Glu Leu Pro Lys Pro Glu Gly Leu Leu Thr Gln Asn Glu Glu Leu
            340                 345                 350
Trp Glu Pro Leu Tyr Ala Cys Tyr Gln Ser Leu Gln Ala Cys Gly Met
        355                 360                 365
Gly Ile Ile Ala Asn Gly Asp Leu Leu Asp Thr Leu Arg Arg Val Lys
    370                 375                 380
Cys Phe Gly Val Pro Leu Val Arg Ile Asp Ile Arg Gln Glu Ser Thr
385                 390                 395                 400
Arg His Thr Glu Ala Leu Gly Glu Leu Thr Arg Tyr Leu Gly Ile Gly
                405                 410                 415
Asp Tyr Glu Ser Trp Ser Glu Ala Asp Lys Gln Ala Phe Leu Ile Arg
```

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                420                 425                 430
Glu Leu Asn Ser Lys Arg Pro Leu Leu Pro Arg Asn Trp Gln Pro Ser
        435                 440                 445
Ala Glu Thr Arg Glu Val Leu Asp Thr Cys Gln Val Ile Ala Glu Ala
    450                 455                 460
Pro Gln Gly Ser Ile Ala Ala Tyr Val Ile Ser Met Ala Lys Thr Pro
465                 470                 475                 480
Ser Asp Val Leu Ala Val His Leu Leu Leu Lys Glu Ala Gly Ile Gly
                485                 490                 495
Phe Ala Met Pro Val Ala Pro Leu Phe Glu Thr Leu Asp Asp Leu Asn
            500                 505                 510
Asn Ala Asn Asp Val Met Thr Gln Leu Leu Asn Ile Asp Trp Tyr Arg
        515                 520                 525
Gly Leu Ile Gln Gly Lys Gln Met Val Met Ile Gly Tyr Ser Asp Ser
    530                 535                 540
Ala Lys Asp Ala Gly Val Met Ala Ala Ser Trp Ala Gln Tyr Gln Ala
545                 550                 555                 560
Gln Asp Ala Leu Ile Lys Thr Cys Glu Lys Ala Gly Ile Glu Leu Thr
                565                 570                 575
Leu Phe His Gly Arg Gly Gly Ser Ile Gly Arg Gly Gly Ala Pro Ala
            580                 585                 590
His Ala Ala Leu Leu Ser Gln Pro Pro Gly Ser Leu Lys Gly Gly Leu
        595                 600                 605
Arg Val Thr Glu Gln Gly Glu Met Ile Arg Phe Lys Tyr Gly Leu Pro
    610                 615                 620
Glu Ile Thr Val Ser Ser Leu Ser Leu Tyr Thr Gly Ala Ile Leu Glu
625                 630                 635                 640
Ala Asn Leu Leu Pro Pro Pro Glu Pro Lys Glu Ser Trp Arg Arg Ile
                645                 650                 655
Met Asp Glu Leu Ser Val Ile Ser Cys Asp Val Tyr Arg Gly Tyr Val
            660                 665                 670
Arg Glu Asn Lys Asp Phe Val Pro Tyr Phe Arg Ser Ala Thr Pro Glu
        675                 680                 685
Gln Glu Leu Gly Lys Leu Pro Leu Gly Ser Arg Pro Ala Lys Arg Arg
    690                 695                 700
Pro Thr Gly Gly Val Glu Ser Leu Arg Ala Ile Pro Trp Ile Phe Ala
705                 710                 715                 720
Trp Thr Gln Asn Arg Leu Met Leu Pro Ala Trp Leu Gly Ala Gly Thr
                725                 730                 735
Ala Leu Gln Lys Val Val Glu Asp Gly Lys Gln Ser Glu Leu Glu Ala
            740                 745                 750
Met Cys Arg Asp Trp Pro Phe Phe Ser Thr Arg Leu Gly Met Leu Glu
        755                 760                 765
Met Val Phe Ala Lys Ala Asp Leu Trp Leu Ala Glu Tyr Tyr Asp Gln
    770                 775                 780
```

```
Arg Leu Val Asp Lys Ala Leu Trp Pro Leu Gly Lys Glu Leu Arg Asn
785                 790                 795                 800
Leu Gln Glu Glu Asp Ile Lys Val Val Leu Ala Ile Ala Asn Asp Ser
                805                 810                 815
His Leu Met Ala Asp Leu Pro Trp Ile Ala Glu Ser Ile Gln Leu Arg
            820                 825                 830
Asn Ile Tyr Thr Asp Pro Leu Asn Val Leu Gln Ala Glu Leu Leu His
        835                 840                 845
Arg Ser Arg Gln Ala Glu Lys Glu Gly Gln Glu Pro Asp Pro Arg Val
    850                 855                 860
Glu Gln Ala Leu Met Val Thr Ile Ala Gly Ile Ala Ala Gly Met Arg
865                 870                 875                 880
Asn Thr Gly
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: other..synthetic DNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCGCGAAGTA GCACCTGTCA CTT 23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: other..synthetic DNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACGGAATTCA ATCTTACGGC C 21

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1643

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Corynebacterium glutamicum
(C) STRAIN: ATCC13869
(ix) FEATURE:
(A) NAME/KEY: mat peptide
(B) LOCATION: 217..1482
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
TCGCGAAGTA GCACCTGTCA CTTTTGTCTC AAATATTAAA TCGAATATCA ATATACGGTC      60
TGTTTATTGG AACGCATCCC AGTGGCTGAG ACGCATCCGC TAAAGCCCCA GGAACCCTGT     120
GCAGAAAGAA AACACTCCTC TGGCTAGGTA GACACAGTTT ATAAAGGTAG AGTTGAGCGG     180
GTAACTGTCA GCACGTAGAT CGAAAGGTGC ACAAAG GTG GCC CTG GTC GTA CAG       234
                                        Met Ala Leu Val Val Gln
                                         1               5

AAA TAT GGC GGT TCC TCG CTT GAG AGT GCG GAA CGC ATT AGA AAC GTC       282
Lys Tyr Gly Gly Ser Ser Leu Glu Ser Ala Glu Arg Ile Arg Asn Val
            10                  15                  20

GCT GAA CGG ATC GTT GCC ACC AAG AAG GCT GGA AAT GAT GTC GTG GTT       330
Ala Glu Arg Ile Val Ala Thr Lys Lys Ala Gly Asn Asp Val Val Val
        25                  30                  35

GTC TGC TCC GCA ATG GGA GAC ACC ACG GAT GAA CTT CTA GAA CTT GCA       378
Val Cys Ser Ala Met Gly Asp Thr Thr Asp Glu Leu Leu Glu Leu Ala
    40                  45                  50

GCG GCA GTG AAT CCC GTT CCG CCA GCT CGT GAA ATG GAT ATG CTC CTG       426
Ala Ala Val Asn Pro Val Pro Pro Ala Arg Glu Met Asp Met Leu Leu
55                  60                  65                  70

ACT GCT GGT GAG CGT ATT TCT AAC GCT CTC GTC GCC ATG GCT ATT GAG       474
Thr Ala Gly Glu Arg Ile Ser Asn Ala Leu Val Ala Met Ala Ile Glu
                75                  80                  85

TCC CTT GGC GCA GAA GCT CAA TCT TTC ACT GGC TCT CAG GCT GGT GTG       522
Ser Leu Gly Ala Glu Ala Gln Ser Phe Thr Gly Ser Gln Ala Gly Val
            90                  95                  100

CTC ACC ACC GAG CGC CAC GGA AAC GCA CGC ATT GTT GAC GTC ACA CCG       570
Leu Thr Thr Glu Arg His Gly Asn Ala Arg Ile Val Asp Val Thr Pro
        105                 110                 115

GGT CGT GTG CGT GAA GCA CTC GAT GAG GGC AAG ATC TGC ATT GTT GCT       618
Gly Arg Val Arg Glu Ala Leu Asp Glu Gly Lys Ile Cys Ile Val Ala
    120                 125                 130

GGT TTT CAG GGT GTT AAT AAA GAA ACC CGC GAT GTC ACC ACG TTG GGT       666
Gly Phe Gln Gly Val Asn Lys Glu Thr Arg Asp Val Thr Thr Leu Gly
135                 140                 145                 150

CGT GGT GGT TCT GAC ACC ACT GCA GTT GCG TTG GCA GCT GCT TTG AAC       714
Arg Gly Gly Ser Asp Thr Thr Ala Val Ala Leu Ala Ala Ala Leu Asn
                155                 160                 165
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GCT GAT GTG TGT GAG ATT TAC TCG GAC GTT GAC GGT GTG TAT ACC GCT      762
Ala Asp Val Cys Glu Ile Tyr Ser Asp Val Asp Gly Val Tyr Thr Ala
            170                 175                 180

GAC CCG CGC ATC GTT CCT AAT GCA CAG AAG CTG GAA AAG CTC AGC TTC      810
Asp Pro Arg Ile Val Pro Asn Ala Gln Lys Leu Glu Lys Leu Ser Phe
        185                 190                 195

GAA GAA ATG CTG GAA CTT GCT GCT GTT GGC TCC AAG ATT TTG GTG CTG      858
Glu Glu Met Leu Glu Leu Ala Ala Val Gly Ser Lys Ile Leu Val Leu
    200                 205                 210

CGC AGT GTT GAA TAC GCT CGT GCA TTC AAT GTG CCA CTT CGC GTA CGC      906
Arg Ser Val Glu Tyr Ala Arg Ala Phe Asn Val Pro Leu Arg Val Arg
215                 220                 225                 230

TCG TCT TAT AGT AAT GAT CCC GGC ACT TTG ATT GCC GGC TCT ATG GAG      954
Ser Ser Tyr Ser Asn Asp Pro Gly Thr Leu Ile Ala Gly Ser Met Glu
                235                 240                 245

GAT ATT CCT GTG GAA GAA GCA GTC CTT ACC GGT GTC GCA ACC GAC AAG     1002
Asp Ile Pro Val Glu Glu Ala Val Leu Thr Gly Val Ala Thr Asp Lys
            250                 255                 260

TCC GAA GCC AAA GTA ACC GTT CTG GGT ATT TCC GAT AAG CCA GGC GAG     1050
Ser Glu Ala Lys Val Thr Val Leu Gly Ile Ser Asp Lys Pro Gly Glu
        265                 270                 275

GCT GCC AAG GTT TTC CGT GCG TTG GCT GAT GCA GAA ATC AAC ATT GAC     1098
Ala Ala Lys Val Phe Arg Ala Leu Ala Asp Ala Glu Ile Asn Ile Asp
    280                 285                 290

ATG GTT CTG CAG AAC GTC TCC TCT GTG GAA GAC GGC ACC ACC GAC ATC     1146
Met Val Leu Gln Asn Val Ser Ser Val Glu Asp Gly Thr Thr Asp Ile
295                 300                 305                 310

ACG TTC ACC TGC CCT CGC GCT GAC GGA CGC CGT GCG ATG GAG ATC TTG     1194
Thr Phe Thr Cys Pro Arg Ala Asp Gly Arg Arg Ala Met Glu Ile Leu
                315                 320                 325

AAG AAG CTT CAG GTT CAG GGC AAC TGG ACC AAT GTG CTT TAC GAC GAC     1242
Lys Lys Leu Gln Val Gln Gly Asn Trp Thr Asn Val Leu Tyr Asp Asp
            330                 335                 340

CAG GTC GGC AAA GTC TCC CTC GTG GGT GCT GGC ATG AAG TCT CAC CCA     1290
Gln Val Gly Lys Val Ser Leu Val Gly Ala Gly Met Lys Ser His Pro
        345                 350                 355

GGT GTT ACC GCA GAG TTC ATG GAA GCT CTG CGC GAT GTC AAC GTG AAC     1338
Gly Val Thr Ala Glu Phe Met Glu Ala Leu Arg Asp Val Asn Val Asn
    360                 365                 370

ATC GAA TTG ATT TCC ACC TCT GAG ATC CGC ATT TCC GTG CTG ATC CGT     1386
Ile Glu Leu Ile Ser Thr Ser Glu Ile Arg Ile Ser Val Leu Ile Arg
375                 380                 385                 390

GAA GAT GAT CTG GAT GCT GCT GCA CGT GCA TTG CAT GAG CAG TTC CAG     1434
Glu Asp Asp Leu Asp Ala Ala Ala Arg Ala Leu His Glu Gln Phe Gln
                395                 400                 405
```

```
CTG GGC GGC GAA GAC GAA GCC GTC GTT TAT GCA GGC ACC GGA CGC TAA      1482
Leu Gly Gly Glu Asp Glu Ala Val Val Tyr Ala Gly Thr Gly Arg
            410                 415                 420

AGTTTTAAAG GAGTAGTTTT ACAATGACCA CCATCGCAGT TGTTGGTGCA ACCGGCCAGG  1542
TCGGCCAGGT TATGCGCACC CTTTTGGAAG AGCGCAATTT CCCAGCTGAC ACTGTTCGTT  1602
TCTTTGCTTC CCCGCGTTCC GCAGGCCGTA AGATTGAATT C                       1643
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 421 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Ala Leu Val Val Gln Lys Tyr Gly Gly Ser Ser Leu Glu Ser Ala
 1               5                  10                  15
Glu Arg Ile Arg Asn Val Ala Glu Arg Ile Val Ala Thr Lys Lys Ala
            20                  25                  30
Gly Asn Asp Val Val Val Val Cys Ser Ala Met Gly Asp Thr Thr Asp
        35                  40                  45
Glu Leu Leu Glu Leu Ala Ala Ala Val Asn Pro Val Pro Pro Ala Arg
    50                  55                  60
Glu Met Asp Met Leu Leu Thr Ala Gly Glu Arg Ile Ser Asn Ala Leu
65                  70                  75                  80
Val Ala Met Ala Ile Glu Ser Leu Gly Ala Glu Ala Gln Ser Phe Thr
                85                  90                  95
Gly Ser Gln Ala Gly Val Leu Thr Thr Glu Arg His Gly Asn Ala Arg
            100                 105                 110
Ile Val Asp Val Thr Pro Gly Arg Val Arg Glu Ala Leu Asp Glu Gly
        115                 120                 125
Lys Ile Cys Ile Val Ala Gly Phe Gln Gly Val Asn Lys Glu Thr Arg
    130                 135                 140
Asp Val Thr Thr Leu Gly Arg Gly Gly Ser Asp Thr Thr Ala Val Ala
145                 150                 155                 160
Leu Ala Ala Ala Leu Asn Ala Asp Val Cys Glu Ile Tyr Ser Asp Val
                165                 170                 175
Asp Gly Val Tyr Thr Ala Asp Pro Arg Ile Val Pro Asn Ala Gln Lys
            180                 185                 190
Leu Glu Lys Leu Ser Phe Glu Glu Met Leu Glu Leu Ala Ala Val Gly
        195                 200                 205
Ser Lys Ile Leu Val Leu Arg Ser Val Glu Tyr Ala Arg Ala Phe Asn
    210                 215                 220
Val Pro Leu Arg Val Arg Ser Ser Tyr Ser Asn Asp Pro Gly Thr Leu
225                 230                 235                 240
```

```
Ile Ala Gly Ser Met Glu Asp Ile Pro Val Glu Glu Ala Val Leu Thr
                245                 250                 255
Gly Val Ala Thr Asp Lys Ser Glu Ala Lys Val Thr Val Leu Gly Ile
            260                 265                 270
Ser Asp Lys Pro Gly Glu Ala Ala Lys Val Phe Arg Ala Leu Ala Asp
        275                 280                 285
Ala Glu Ile Asn Ile Asp Met Val Leu Gln Asn Val Ser Ser Val Glu
    290                 295                 300
Asp Gly Thr Thr Asp Ile Thr Phe Thr Cys Pro Arg Ala Asp Gly Arg
305                 310                 315                 320
Arg Ala Met Glu Ile Leu Lys Lys Leu Gln Val Gln Gly Asn Trp Thr
                325                 330                 335
Asn Val Leu Tyr Asp Asp Gln Val Gly Lys Val Ser Leu Val Gly Ala
            340                 345                 350
Gly Met Lys Ser His Pro Gly Val Thr Ala Glu Phe Met Glu Ala Leu
        355                 360                 365
Arg Asp Val Asn Val Asn Ile Glu Leu Ile Ser Thr Ser Glu Ile Arg
    370                 375                 380
Ile Ser Val Leu Ile Arg Glu Asp Asp Leu Asp Ala Ala Ala Arg Ala
385                 390                 395                 400
Leu His Glu Gln Phe Gln Leu Gly Gly Glu Asp Glu Ala Val Val Tyr
                405                 410                 415
Ala Gly Thr Gly Arg
            420
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1643

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Corynebacterium glutamicum

(C) STRAIN: ATCC13869

(ix) FEATURE:

(A) NAME/KEY: mat peptide

(B) LOCATION: 964..1482

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
TCGCGAAGTA GCACCTGTCA CTTTTGTCTC AAATATTAAA TCGAATATCA ATATACGGTC     60
TGTTTATTGG AACGCATCCC AGTGGCTGAG ACGCATCCGC TAAAGCCCCA GGAACCCTGT    120
GCAGAAAGAA AACACTCCTC TGGCTAGGTA GACACAGTTT ATAAAGGTAG AGTTGAGCGG    180
GTAACTGTCA GCACGTAGAT CGAAAGGTGC ACAAAGGTGG CCCTGGTCGT ACAGAAATAT    240
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```
GGCGGTTCCT CGCTTGAGAG TGCGGAACGC ATTAGAAACG TCGCTGAACG GATCGTTGCC    300
ACCAAGAAGG CTGGAAATGA TGTCGTGGTT GTCTGCTCCG CAATGGGAGA CACCACGGAT    360
GAACTTCTAG AACTTGCAGC GGCAGTGAAT CCCGTTCCGC CAGCTCGTGA AATGGATATG    420
CTCCTGACTG CTGGTGAGCG TATTTCTAAC GCTCTCGTCG CCATGGCTAT TGAGTCCCTT    480
GGCGCAGAAG CTCAATCTTT CACTGGCTCT CAGGCTGGTG TGCTCACCAC CGAGCGCCAC    540
GGAAACGCAC GCATTGTTGA CGTCACACCG GGTCGTGTGC GTGAAGCACT CGATGAGGGC    600
AAGATCTGCA TTGTTGCTGG TTTTCAGGGT GTTAATAAAG AAACCCGCGA TGTCACCACG    660
TTGGGTCGTG GTGGTTCTGA CACCACTGCA GTTGCGTTGG CAGCTGCTTT GAACGCTGAT    720
GTGTGTGAGA TTTACTCGGA CGTTGACGGT GTGTATACCG CTGACCCGCG CATCGTTCCT    780
AATGCACAGA AGCTGGAAAA GCTCAGCTTC GAAGAAATGC TGGAACTTGC TGCTGTTGGC    840
TCCAAGATTT TGGTGCTGCG CAGTGTTGAA TACGCTCGTG CATTCAATGT GCCACTTCGC    900
GTACGCTCGT CTTATAGTAA TGATCCCGGC ACTTTGATTG CCGGCTCTAT GGAGGATATT    960
CCT  GTG GAA GAA GCA GTC CTT ACC GGT GTC GCA ACC GAC AAG TCC GAA     1008
     Met Glu Glu Ala Val Leu Thr Gly Val Ala Thr Asp Lys Ser Glu
       1               5                  10                  15
GCC AAA GTA ACC GTT CTG GGT ATT TCC GAT AAG CCA GGC GAG GCT GCC     1056
Ala Lys Val Thr Val Leu Gly Ile Ser Asp Lys Pro Gly Glu Ala Ala
                20                  25                  30
AAG GTT TTC CGT GCG TTG GCT GAT GCA GAA ATC AAC ATT GAC ATG GTT     1104
Lys Val Phe Arg Ala Leu Ala Asp Ala Glu Ile Asn Ile Asp Met Val
            35                  40                  45
CTG CAG AAC GTC TCC TCT GTG GAA GAC GGC ACC ACC GAC ATC ACG TTC     1152
Leu Gln Asn Val Ser Ser Val Glu Asp Gly Thr Thr Asp Ile Thr Phe
        50                  55                  60
ACC TGC CCT CGC GCT GAC GGA CGC CGT GCG ATG GAG ATC TTG AAG AAG     1200
Thr Cys Pro Arg Ala Asp Gly Arg Arg Ala Met Glu Ile Leu Lys Lys
    65                  70                  75
CTT CAG GTT CAG GGC AAC TGG ACC AAT GTG CTT TAC GAC GAC CAG GTC     1248
Leu Gln Val Gln Gly Asn Trp Thr Asn Val Leu Tyr Asp Asp Gln Val
80                  85                  90                  95
GGC AAA GTC TCC CTC GTG GGT GCT GGC ATG AAG TCT CAC CCA GGT GTT     1296
Gly Lys Val Ser Leu Val Gly Ala Gly Met Lys Ser His Pro Gly Val
                100                 105                 110
ACC GCA GAG TTC ATG GAA GCT CTG CGC GAT GTC AAC GTG AAC ATC GAA     1344
Thr Ala Glu Phe Met Glu Ala Leu Arg Asp Val Asn Val Asn Ile Glu
            115                 120                 125
TTG ATT TCC ACC TCT GAG ATC CGC ATT TCC GTG CTG ATC CGT GAA GAT     1392
Leu Ile Ser Thr Ser Glu Ile Arg Ile Ser Val Leu Ile Arg Glu Asp
        130                 135                 140
GAT CTG GAT GCT GCT GCA CGT GCA TTG CAT GAG CAG TTC CAG CTG GGC     1440
Asp Leu Asp Ala Ala Ala Arg Ala Leu His Glu Gln Phe Gln Leu Gly
    145                 150                 155
GGC GAA GAC GAA GCC GTC GTT TAT GCA GGC ACC GGA CGC TAAAGTTTTAA     1490
Gly Glu Asp Glu Ala Val Val Tyr Ala Gly Thr Gly Arg
160                 165                 170     172
```

AGGAGTAGTT TTACAATGAC CACCATCGCA GTTGTTGGTG CAACCGGCCA GGTCGGCCAG 1550
GTTATGCGCA CCCTTTTGGA AGAGCGCAAT TTCCCAGCTG ACACTGTTCG TTTCTTTGCT 1610
TCCCCGCGTT CCGCAGGCCG TAAGATTGAA TTC 1643

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 172 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Glu Glu Ala Val Leu Thr Gly Val Ala Thr Asp Lys Ser Glu Ala
1 5 10 15
Lys Val Thr Val Leu Gly Ile Ser Asp Lys Pro Gly Glu Ala Ala Lys
20 25 30
Val Phe Arg Ala Leu Ala Asp Ala Glu Ile Asn Ile Asp Met Val Leu
35 40 45
Gln Asn Val Ser Ser Val Glu Asp Gly Thr Thr Asp Ile Thr Phe Thr
50 55 60
Cys Pro Arg Ala Asp Gly Arg Arg Ala Met Glu Ile Leu Lys Lys Leu
65 70 75 80
Gln Val Gln Gly Asn Trp Thr Asn Val Leu Tyr Asp Asp Gln Val Gly
85 90 95
Lys Val Ser Leu Val Gly Ala Gly Met Lys Ser His Pro Gly Val Thr
100 105 110
Ala Glu Phe Met Glu Ala Leu Arg Asp Val Asn Val Asn Ile Glu Leu
115 120 125
Ile Ser Thr Ser Glu Ile Arg Ile Ser Val Leu Ile Arg Glu Asp Asp
130 135 140
Leu Asp Ala Ala Ala Arg Ala Leu His Glu Gln Phe Gln Leu Gly Gly
145 150 155 160
Glu Asp Glu Ala Val Val Tyr Ala Gly Thr Gly Arg
165 170

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: other..synthetic DNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAAAACCTGC GTTCTC 16

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULAR TYPE: other..synthetic DNA
(iii) HYPOTHETICAL: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
TGACTTAAG GTTTACAGGCC 20

(2) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULAR TYPE: other..synthetic DNA
(iii) HYPOTHETICAL: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
ACTGAATTCC AAATGTCCGC 20

(2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULAR TYPE: other..synthetic DNA
(iii) HYPOTHETICAL: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:
AAGTGCAGG CCGTTT 16

Claims

1. A mutant phosphoenolpyruvate carboxylase originating from a microorganism belonging to the genus Escherichia, wherein said mutant phosphoenolpyruvate carboxylase has mutation to desensitize feedback inhibition of the phosphoenolpyruvate carboxylase by aspartic acid.

2. A mutant phosphoenolpyruvate carboxylase according to claim 1, which, in the case of being allowed to exist in cells of a microorganism belonging to the genus Escherichia, gives the cells resistance to a compound having the following properties:

it exhibits a growth inhibitory action against a microorganism belonging to the genus Escherichia which produces a wild type phosphoenolpyruvate carboxylase;

said growth inhibitory action is recovered by existence of L-glutamic acid or L-aspartic acid; and

it inhibits wild type phosphoenolpyruvate carboxylase activity.

3. A mutant phosphoenolpyruvate carboxylase according to claim 2, wherein said compound is selected from 3-bromopyruvate, aspartic acid-β-hydrazide and DL-threo-β-hydroxyaspartic acid.

4. A mutant phosphoenolpyruvate carboxylase according to claim 1, wherein said mutation is mutation to replace 625th glutamic acid with lysine as counted from the N-terminus of the phosphoenolpyruvate carboxylase.

5. A mutant phosphoenolpyruvate carboxylase according to claim 1, wherein said mutation is mutation to replace 222th arginine with histidine and 223th glutamic acid with lysine respectively as counted from the N-terminus of the phosphoenolpyruvate carboxylase.

6. A mutant phosphoenolpyruvate carboxylase according to claim 1, wherein said mutation is mutation to replace 288th serine with phenylalanine, 289th glutamic acid with lysine, 551th methionine with isoleucine and 804th glutamic acid with lysine respectively as counted from the N-terminus of the phosphoenolpyruvate carboxylase.

7. A mutant phosphoenolpyruvate carboxylase according to claim 1, wherein said mutation is mutation to replace 867th alanine with threonine as counted from the N-terminus of the phosphoenolpyruvate carboxylase.

8. A mutant phosphoenolpyruvate carboxylase according to claim 1, wherein said mutation is mutation to replace 438th arginine with cysteine as counted from the N-terminus of the phosphoenolpyruvate carboxylase.

9. A mutant phosphoenolpyruvate carboxylase according to claim 1, wherein said mutation is mutation to replace 620th lysine with serine as counted from the N-terminus of the phosphoenolpyruvate carboxylase.

10. A DNA fragment which codes for the mutant phosphoenolpyruvate carboxylase according to any one of claims 1 to 9.

11. A microorganism belonging to the genus Escherichia or coryneform bacteria, transformed by allowing the DNA fragment according to claim 10 to be integrated in chromosomal DNA.

12. A recombinant DNA formed by ligating the DNA fragment according to claim 10 with a vector DNA capable of autonomously replication in cells of bacteria belonging to the genus Escherichia or coryneform bacteria.

13. A microorganism belonging to the genus Escherichia or coryneform bacteria, transformed with the recombinant DNA according to claim 12.

14. A method of producing amino acid, comprising:
cultivating the microorganism according to claim 11 or 13 in a suitable medium; and
separating, from the medium, an amino acid selected from the group consisting of L-lysine, L-threonine, L-methionine, L-isoleucine, L-glutamic acid, L-arginine and L-proline.

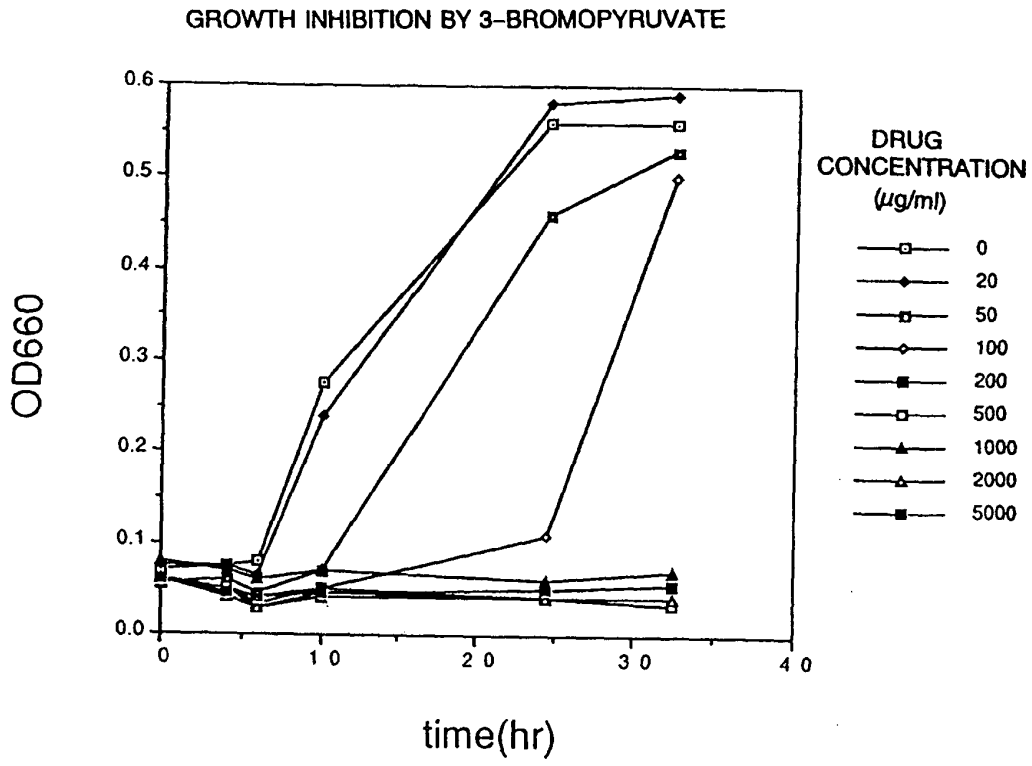

GROWTH INHIBITION BY 3-BROMOPYRUVATE
OD660
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
time(hr)
DRUG CONCENTRATION (μg/ml)
0
20
50
100
200
500
1000
2000
5000

Fig. 1

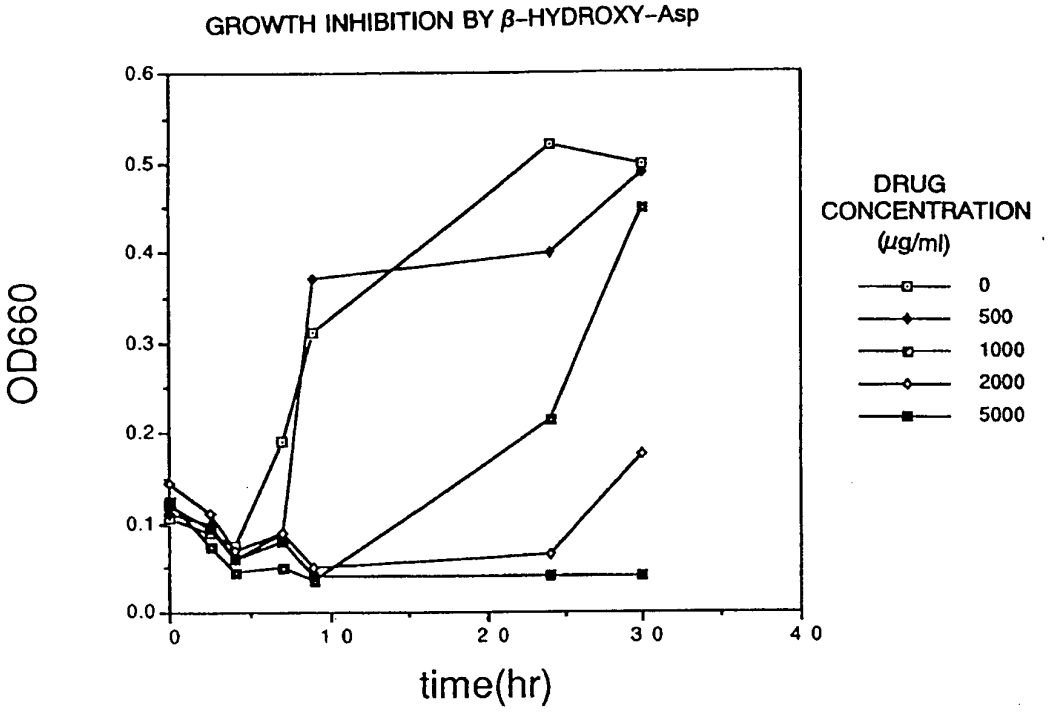

GROWTH INHIBITION BY β-HYDROXY-Asp
OD660
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
time(hr)
DRUG CONCENTRATION (μg/ml)
0
500
1000
2000
5000

Fig. 2

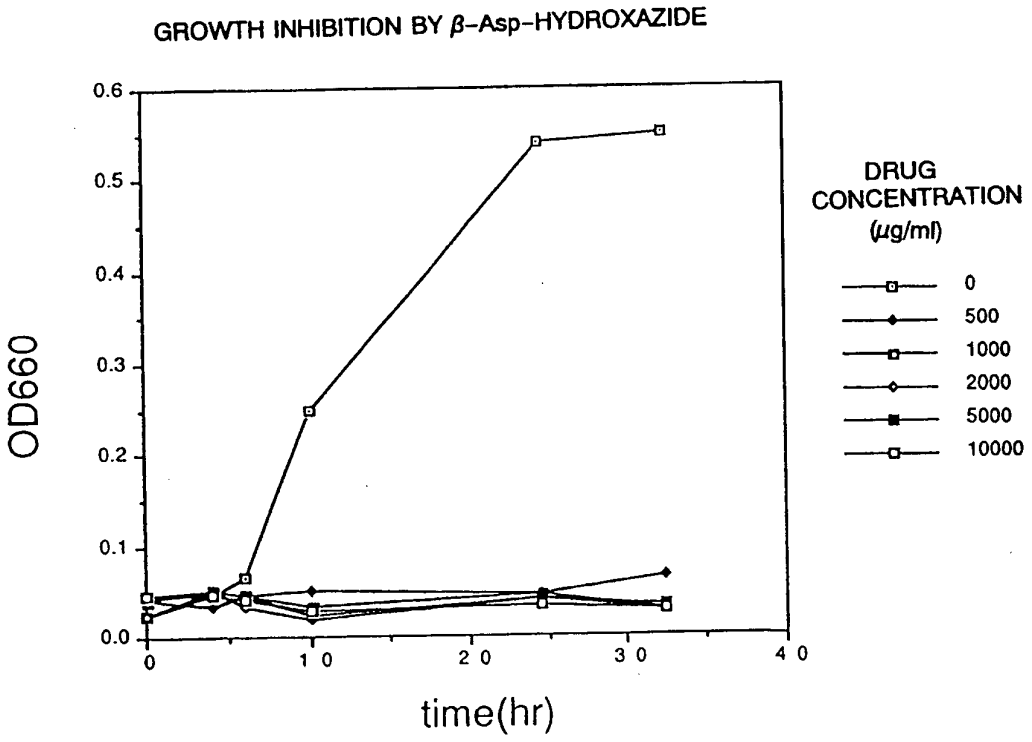

GROWTH INHIBITION BY β-Asp-HYDROXAZIDE
OD660
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
time(hr)
DRUG CONCENTRATION (μg/ml)
0
500
1000
2000
5000
10000

Fig. 3

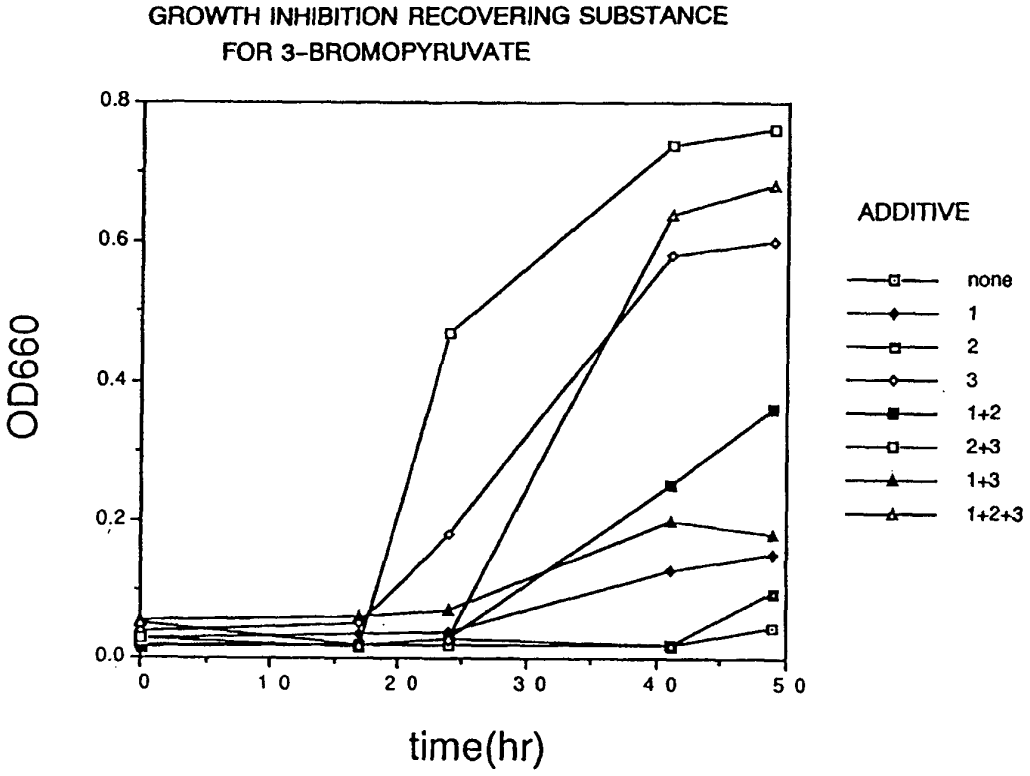

GROWTH INHIBITION RECOVERING SUBSTANCE
FOR 3-BROMOPYRUVATE
OD660
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
time(hr)
ADDITIVE
none
1
2
3
1+2
2+3
1+3
1+2+3

Fig. 4

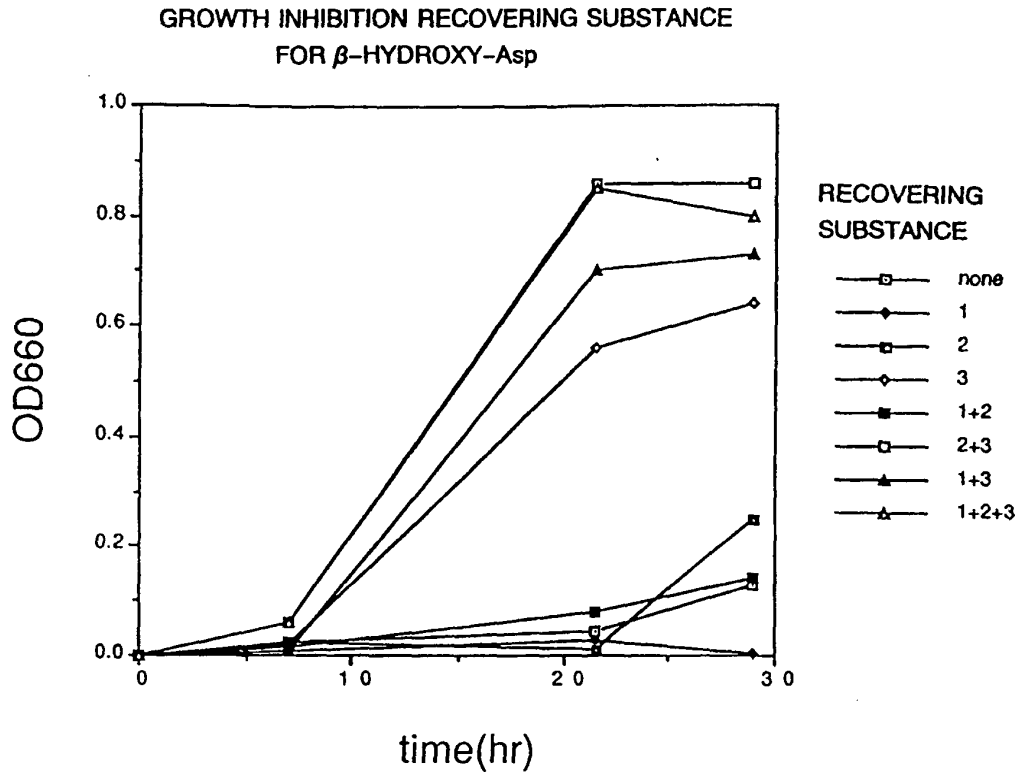

GROWTH INHIBITION RECOVERING SUBSTANCE
FOR β-HYDROXY-Asp
OD660
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
time(hr)
RECOVERING SUBSTANCE
none
1
2
3
1+2
2+3
1+3
1+2+3

Fig. 5

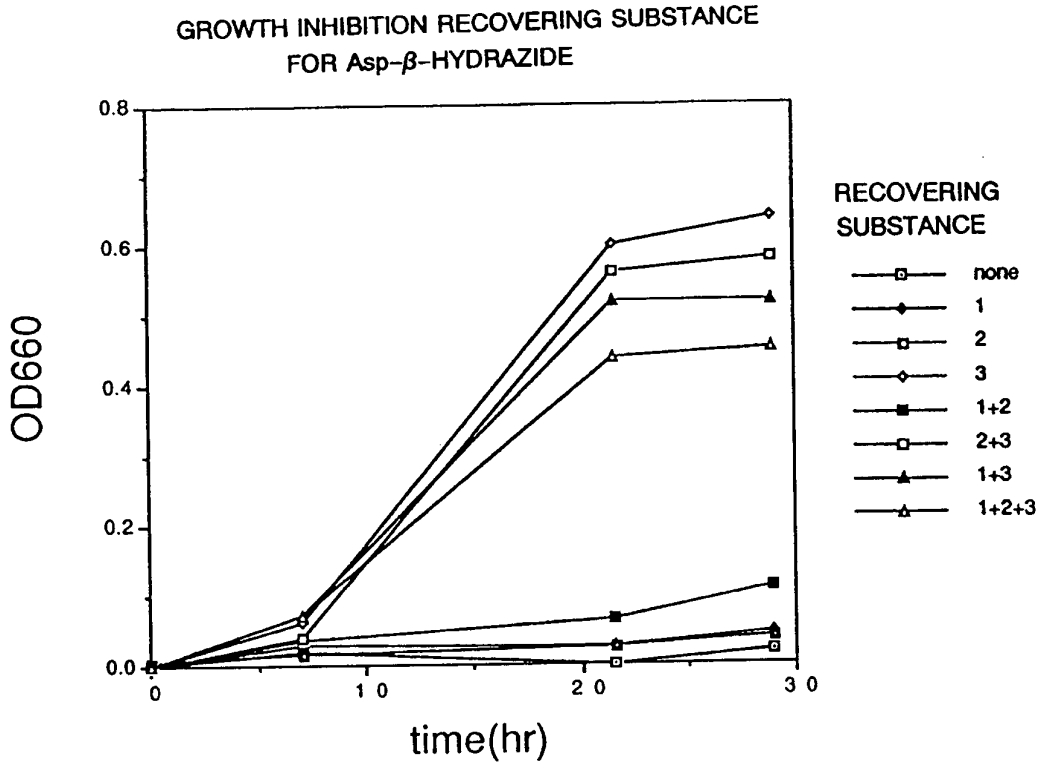

GROWTH INHIBITION RECOVERING SUBSTANCE
FOR Asp-β-HYDRAZIDE
OD660
0.8
0.6
0.4
0.2
0.0
0
10
20
30
time(hr)
RECOVERING
SUBSTANCE
none
1
2
3
1+2
2+3
1+3
1+2+3

Fig. 6

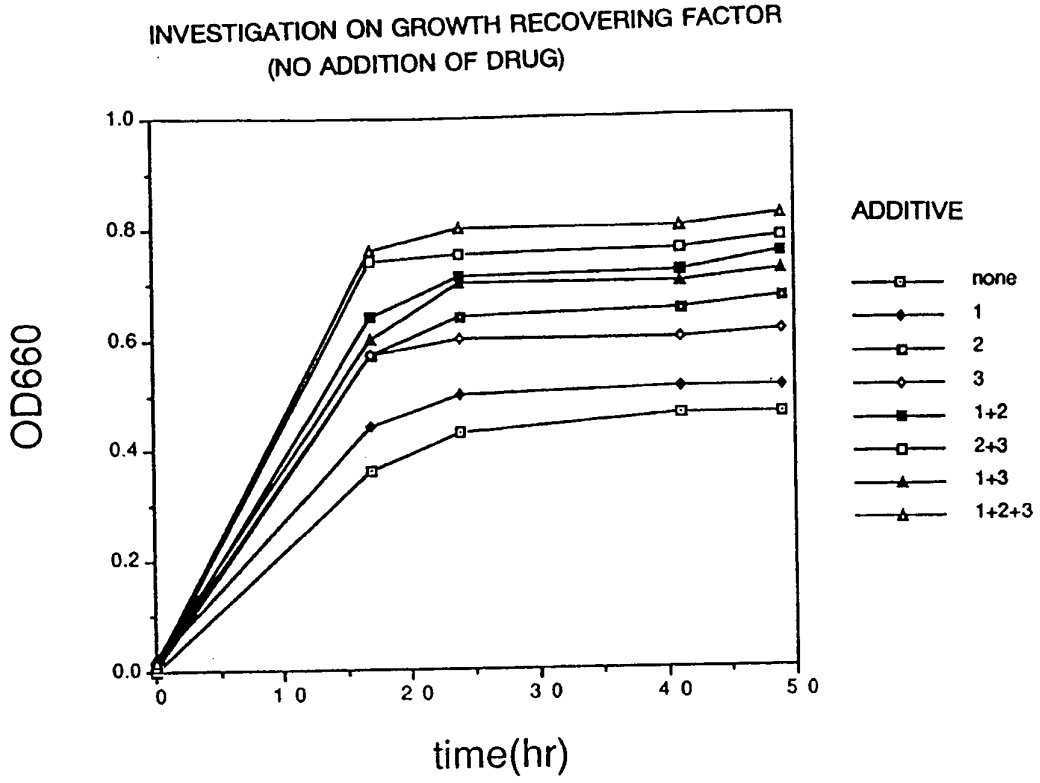

INVESTIGATION ON GROWTH RECOVERING FACTOR
(NO ADDITION OF DRUG)
OD660
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
time(hr)
ADDITIVE
none
1
2
3
1+2
2+3
1+3
1+2+3

Fig. 7

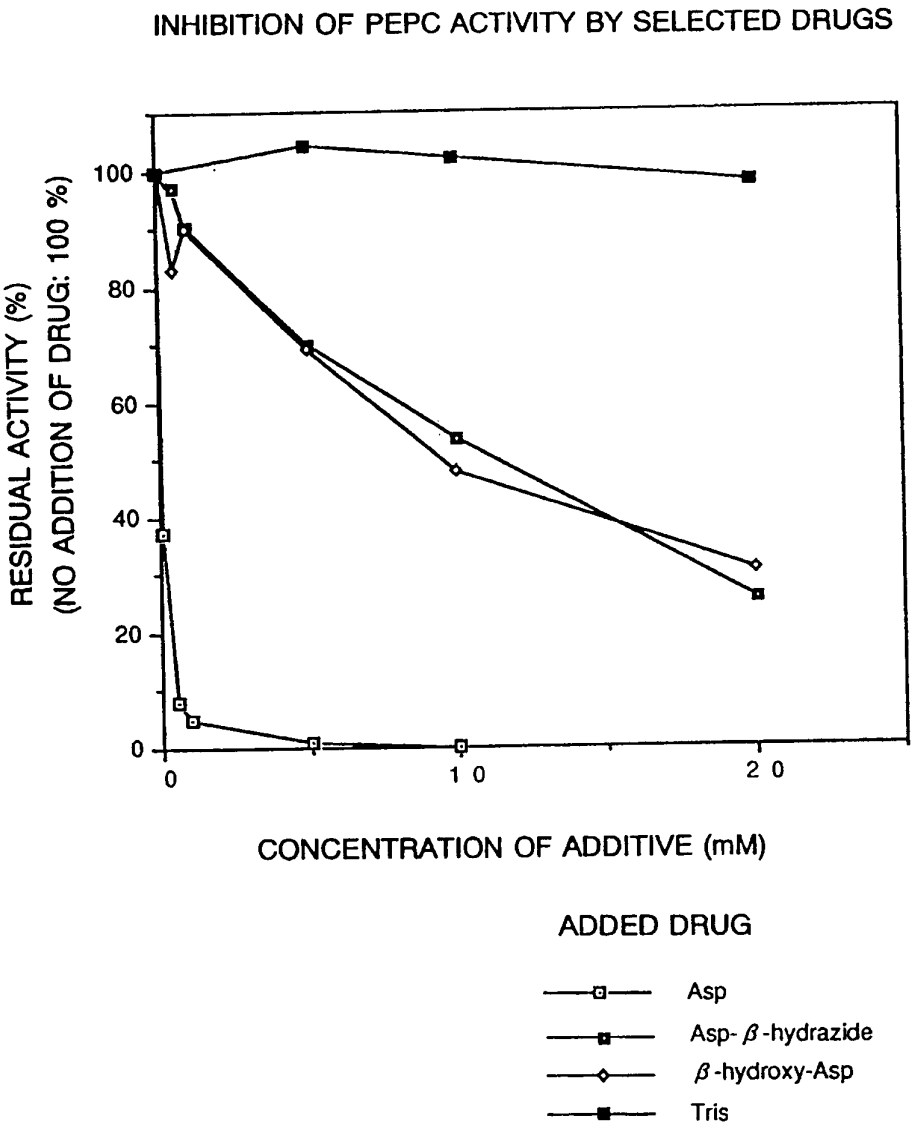

INHIBITION OF PEPC ACTIVITY BY SELECTED DRUGS
RESIDUAL ACTIVITY (%)
(NO ADDITION OF DRUG: 100 %)
100
80
60
40
20
0
0
10
20
CONCENTRATION OF ADDITIVE (mM)
ADDED DRUG
Asp
Asp-β-hydrazide
β-hydroxy-Asp
Tris

Fig. 8

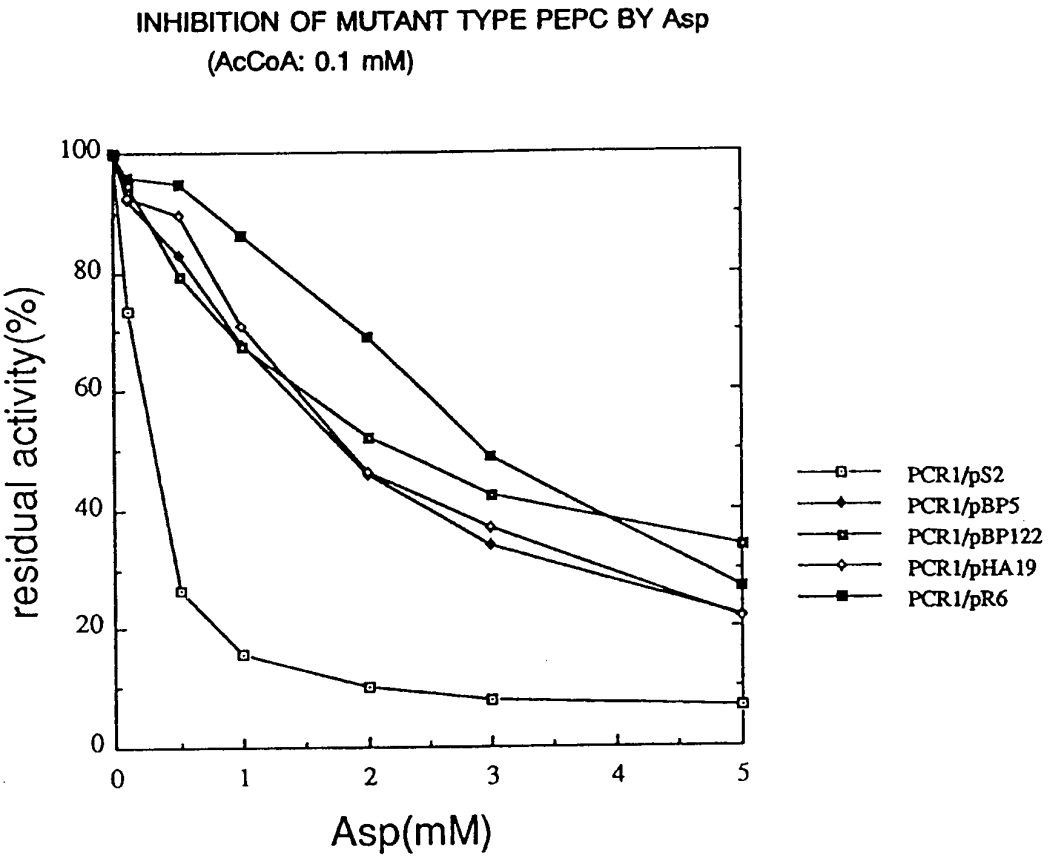

INHIBITION OF MUTANT TYPE PEPC BY Asp
(AcCoA: 0.1 mM)
residual activity(%)
100
80
60
40
20
0
0
1
2
3
4
5
Asp(mM)
PCR1/pS2
PCR1/pBP5
PCR1/pBP122
PCR1/pHA19
PCR1/pR6

Fig. 9

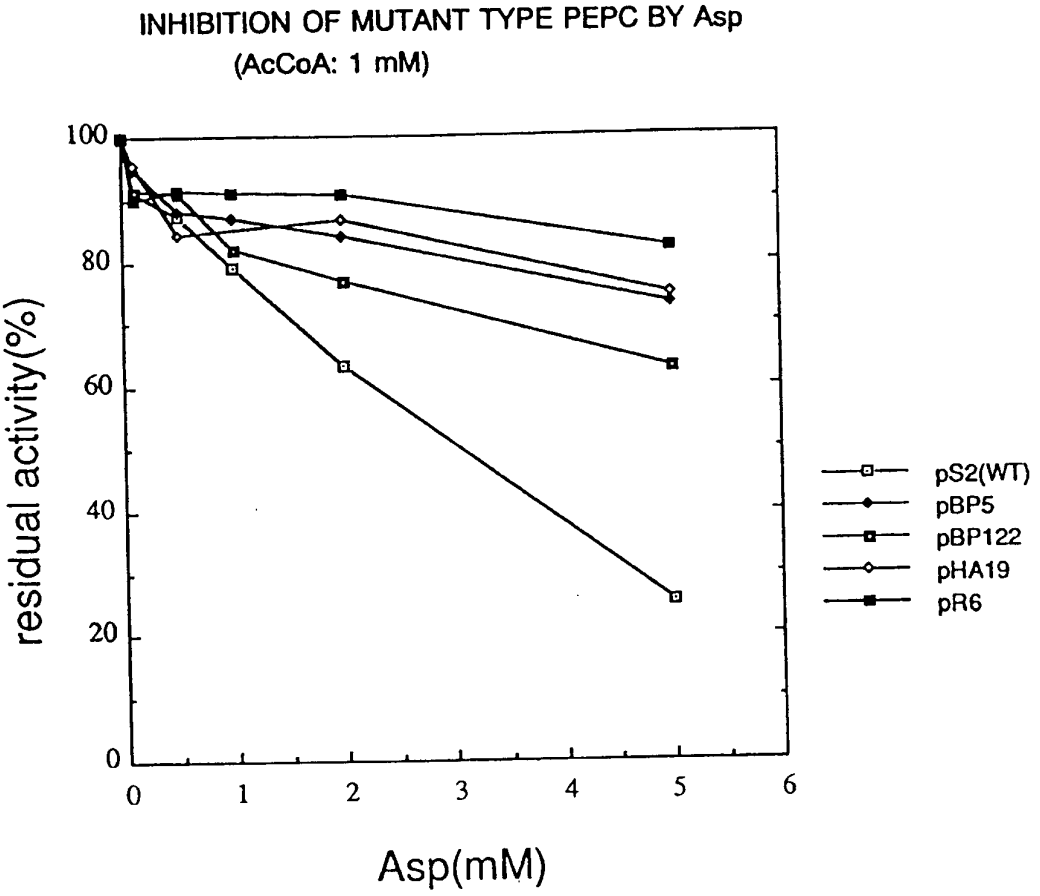

INHIBITION OF MUTANT TYPE PEPC BY Asp
(AcCoA: 1 mM)
residual activity(%)
100
80
60
40
20
0
0
1
2
3
4
5
6
Asp(mM)
pS2(WT)
pBP5
pBP122
pHA19
pR6

Fig. 10

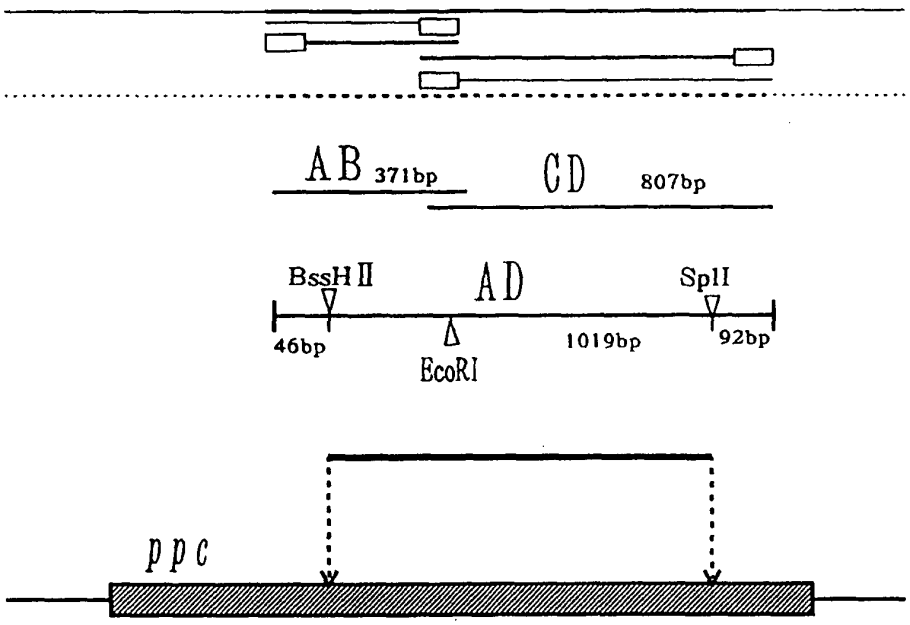
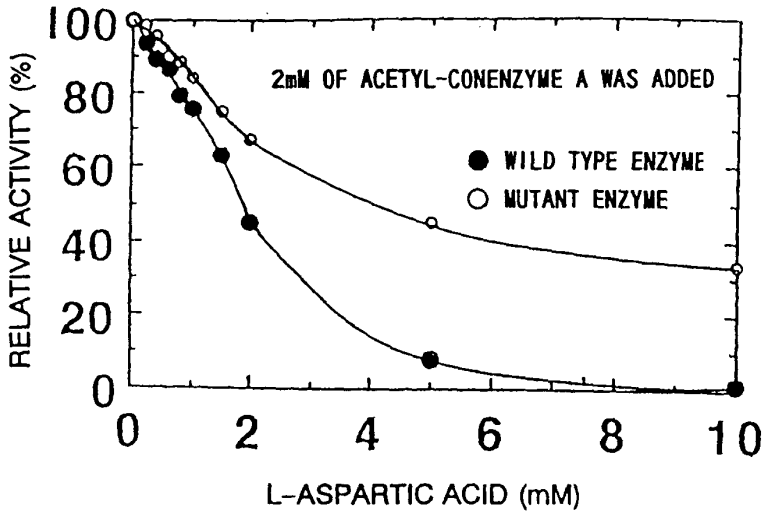


Fig. 11


2mM OF ACETYL-CONENZYME A WAS ADDED
WILD TYPE ENZYME
MUTANT ENZYME
RELATIVE ACTIVITY (%)
100
80
60
40
20
0
0
2
4
6
8
10
L-ASPARTIC ACID (mM)

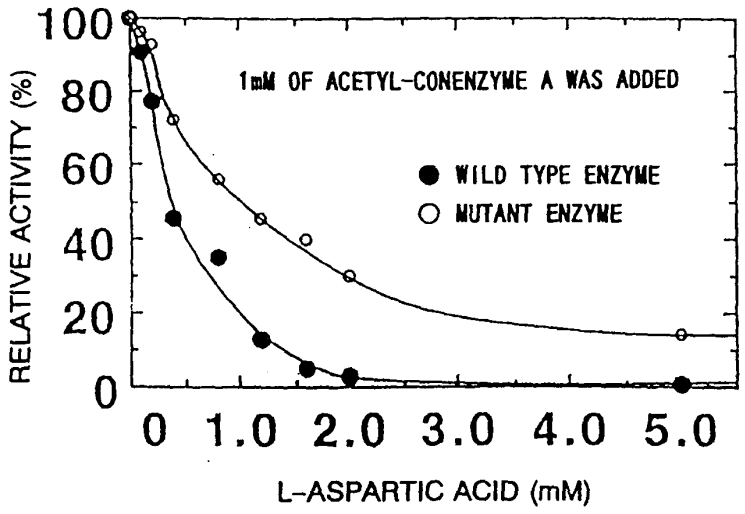

1mM OF ACETYL-CONENZYME A WAS ADDED
WILD TYPE ENZYME
MUTANT ENZYME
RELATIVE ACTIVITY (%)
100
80
60
40
20
0
0
1.0
2.0
3.0
4.0
5.0
L-ASPARTIC ACID (mM)

Fig. 12

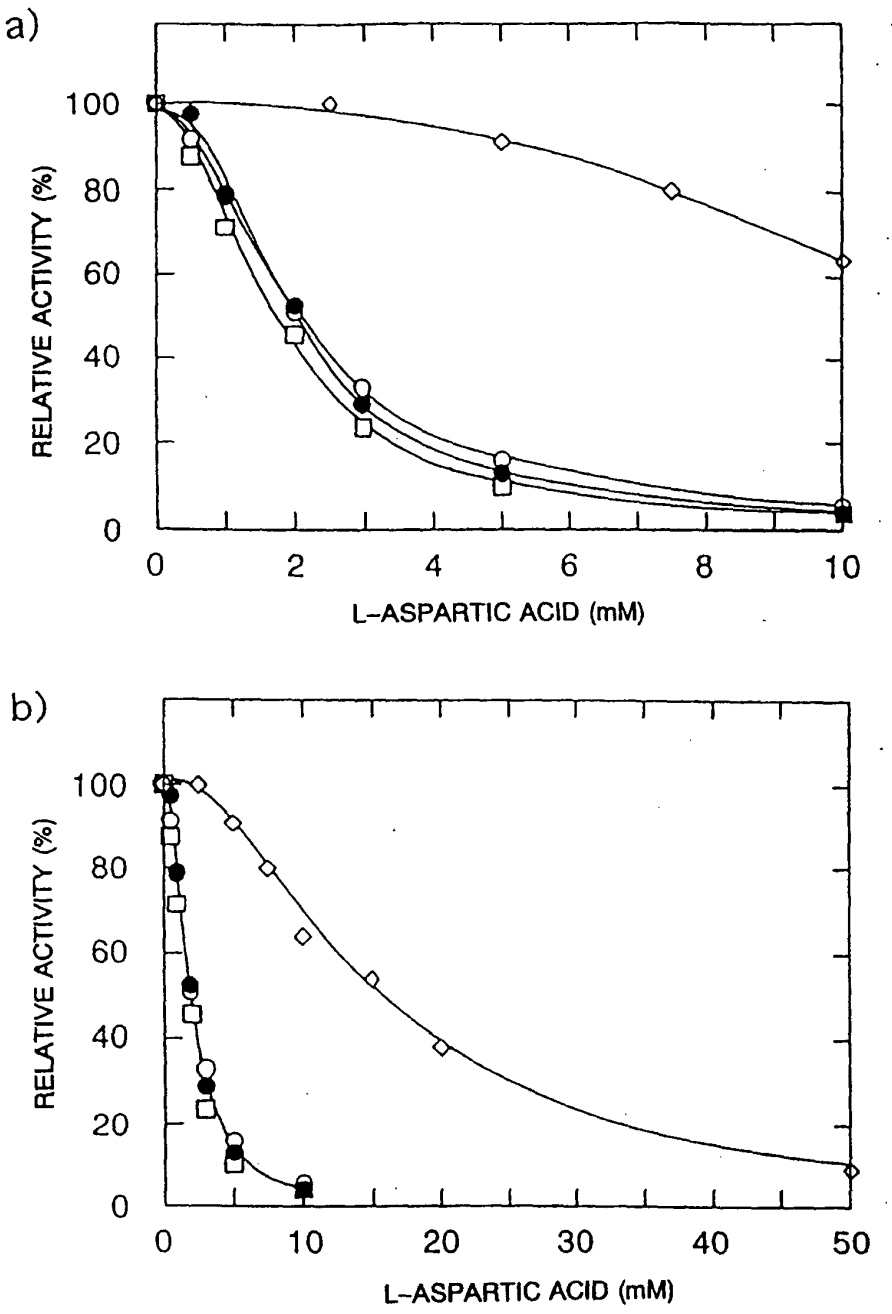

a)
RELATIVE ACTIVITY (%)
100
80
60
40
20
0
0
2
4
6
8
10
L-ASPARTIC ACID (mM)
b)
RELATIVE ACTIVITY (%)
100
80
60
40
20
0
0
10
20
30
40
50
L-ASPARTIC ACID (mM)
WILD TYPE ENZYME (●)
K650A MUTANT ENZYME (○)
K491A MUTANT ENZYME (□)
K620S MUTANT ENZYME (◇)

Fig. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP94/01365

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl^6 C12N9/88

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl^5 C12N9/88, C12N15/60

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS BIOSIS WPI, WPI/L

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Agric Biol Chem. Vol. 47, No. 7 (1983), Hachiro Ozaki et al. "Production of lysine by pyruvate kinase mutants of Brevibacterium flavum", P. 1569-1576	1, 2, 14
A	J. Biochem. Vol. 95, No. 4 (1984), Fujita Nubuuki et al. "The Primary structure of phosphoenolpyruvate carboxylase of Escherichia coli Nucleotide Sequence of the ppe gene and deduced aminoacid Sequence", P. 909-916	4-13
A	J. Biol Chem. Vol. 265, No. 26 (1990), Sherryl Mowbray et al. "Mutations in the Aspartate Receptor of Escherichia coli Which Affect Aspartate Binding", P. 15638-15643	8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
November 1, 1994 (01. 11. 94)	November 22, 1994 (22. 11. 94)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)